A study on virus diseases of parsnip (Pastinaca sativa L.)

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Award date:
1990

Awarding institution:
University of Bath

A STUDY ON VIRUS DISEASES OF PARSNIP (*Pastinaca sativa* L)

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FOR THE DEGREE OF PhD

OF THE UNIVERSITY OF BATH

1990.

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Acknowledgements

I wish to express my sincere gratitude to Dr. R.G.T. Hicks for his supervision and guidance throughout this project.

I also thank Dr. D.G.A. Walkey, Institute of Horticultural Reseach, Wellesbourne; for supplying parsnip yellow fleck virus antiserum, and Dr. A.F. Murant, Scottish Horticultural Research Institute, Dundee; for supplying strawberry latent ringspot virus antiserum.

I also wish to thank Mr. Tim. Moxham, Mr.Chris Vennard, Mr. P. Clark, of Department of Plant Sciences, and Mr. Paul Maggs of the Department of Microbiology for their technical assistance. The help given by the glasshouse staff at Bath University is also gratefully acknowledged.

I am indebted to the Commonwealth Scholarship Commission for its financial support towards this project.

I also wish to express gratitute to my friend, Mr. Karl Morten for the help he has given me.

Finally, I thank the Department of Botany of my home University, Sri Jayewardenepura, Sri Lanka for their invaluable encouragement.

Abstract

A small survey of 17 field-grown parsnips cv. White Gem plants with chlorotic mottling and flecking symptoms, detected two viruses. The nepovirus, strawberry latent ringspot virus (SLRV), was isolated from seven plants and parsnip yellow fleck virus (PYFV) was isolated from one plant.

Strawberry latent ringspot virus was isolated from both leaves and roots but concentrations of virus were apparently lower in leaves than in roots. Leaf extracts were found to contain more of the substances that inhibited the infection of, ***Phaseolus vulgaris*** by tobacco necrosis virus.

Isolates of SLRV differed in their host range and reaction and could be grouped according to their virulence in test plants. Three isolates of SLRV which were of 'mild', 'intermediate', or 'severe' virulence in ***Chenopodium quinoa,*** were studied further. Apart from some differences in host range and reaction, these isolates resembled in physical properties and particle morphology, isolates of SLRV described from other hosts.

Isolates of SLRV from parsnip were seed transmitted in several hosts, where transmission often exceeded 75%. In cucumber, seed infection was shown to arise through infection of either the male or female

gametes.

Controlled pore glass chromatography was used to purify two isolates of SLRV and to prepare antiserum. Little or no serological difference was found between the parsnip isolates or between these and an isolate from *Caryopteris clandonensis*. Slight differences were detected between the isolates in their electrophoretic mobility. The relative molecular masses (Mr) of the capsid proteins for SLRV isolates were similar to published data.

Enzyme-linked immunosorbent assay and double-stranded RNA (dsRNA) analysis was used to detect virus in parsnip and other experimental hosts. Analysis of double-stranded RNA extracted from SLRV-infected tissues revealed only minor differences in band pattern. Less dsRNA was extracted from parsnip leaves than roots, and from plants grown at higher rather than lower post-inoculation temperatures.

An isolate of SLRV from parsnip was successfully inoculated to apparently healthy parsnip seedlings, and chlorotic symptoms were observed. Transmission to parsnips was demonstrated using nematode, graft and mechanical inoculation techniques. The effect of temperature and light on some of these procedures was investigated.

A combination of seed culture and thermotherapy resulted in the production of apparently virus-free cultures or plants of *Nicotiana rustica, Chenopodium quinoa,* and *Pastinaca sativa,* previously infected with SLRV. Growth of infected seedlings on medium containing ribavirin resulted in some virus-free cultures.

A single isolate of PYFV was isolated from parsnip and characterised with respect to host range and reaction, physical and chemical properties. A limited comparison between this isolate and an isolate of PYFV from Angelica, and published data on other parsnip isolates of PYFV, showed few differences.

Low levels of seed transmission of PYFV in *N. clevelandii* were found, a first record for this virus. Some evidence for synergism was obtained in test plants infected with both PYFV and SLRV, with respect to symptom expression.

ABBREVIATIONS

A	= Absorbance (at stated wavelength)
ALP	= Alkaline phosphotase
AMV	= *Arabis* mosaic virus
AYV	= *Anthriscus* yellows virus
BSA	= bovine serum albumin
CA	= carbonic anhydrase
CB	= carbonate buffer
CPG	= controll^{ed} pore glass
cv	= cultivar
DEP	= dilution end-point
DNase	= deoxyribonuclease
dsRNA	= double-stranded RNA
DYMV	= dandelion yellow mosaic virus
E	= extinction coefficient
EA	= egg albumin
ELISA	= enzyme-linked immunosorbent assay
GPD	= glyceraldehyde-3-phosphate dehydrogenase
h	= hours
IgG	= gamma-globulin
LA	= lactalbumin
LIV	= longevity in vitro
min.	= minutes
Mr	= relative molecular mass
N	= nitrogen
p	= phosphorus
PBS	= phosphate buffered saline
PEG	= polyethylene glycol
PTA	= *dodeca* tungstophosphoric acid
PLRV	= potato leaf roll virus
PVX	= potato virus X
PVY	= potato virus Y
PYFV	= parsnip yellow fleck virus
K	= potash
RNase	= ribonuclease
RRV	= raspberry ringspot virus
SDS	= sodium lauril sulphate
SLRV	= strawberry latent ringspot virus
spp.	= species
ssRNA	= single-stranded RNA
TG	= trypsinogen
TI	= trypsin inhibitor
TIP	= thermal inactivation point
TNV	= tobacco necrosis virus
uv	= ultra violet

CHAPTER 1 INTRODUCTION

Strawberry latent ringspot virus (SLRV)

Strawberry latent ringspot virus is found naturally in a range of cultivated and wild plants in Western Europe including Great Britain (Lister, 1964 ; Schmelzer, 1969). It has also been found once in New Zealand (Fry and Wood, 1973) and Canada (Allen ***et al.,*** 1970) in imported planting materials of sweet cherry, and once in the United States (Hanson and Campbell, 1979) in cow parsley. The virus has a wide host range and it has been reported that out of 167 species belonging to dicotyledenous plants inoculated mechanically, the virus infected 126 belonging to 27 families (Schmelzer, 1969) including the family Umbelliferae. Natural infection with SLRV has been reported for several umbelliferous crops including celery (Walkey and Mitchell, 1969) and parsnip (Van der Put, 1979, Cooper, 1981)

Parsnip (***Pastinaca sativa*** L. syn.***Peucedanum sativum***) is widely grown in temperate countries as a vegetable crop, and is known to have been used for food in Britain during the time of the second Roman Occupation. It is a biennial plant and has a nutritious root, marketed as a vegetable at the end of the first growing season. The root is rich in starch and sugar and is of economic importance especially in temperate regions. Parsnips are known to be susceptible to several viruses other than SLRV

including celery mosaic (Buterac *et al.*, 1974), parsnip mosaic (Murant, 1972), parsnip mottle (Watson *et al.*, 1964) and parsnip yellow fleck (Murant and Goold, 1968) which are all aphid-borne.

Van der Put (1979) in the Netherlands was the first to detect SLRV in parsnip although identification was based only on the physical properties and the nature of particles under the electron microscope.

The first report of SLRV infection in parsnip in the United Kingdom was in 1981, when Cooper (1981) isolated it from seedlings with slight chlorotic leaf mottle, in Northern Ireland. The virus was detected by sap transmission to *Chenopodium* spp., electron microscopy, physical properties and was confirmed by serology.

In 1986, Hicks *et al.*, reported the isolation of SLRV from parsnip cv. White Gem in a seed crop at Bath University Field Station. The virus was characterised by host range, physical properties, serology and electron microscopy. These authors described 'severe' and 'mild' isolates of SLRV from parsnips with respect to their biological and physical properties.

Strawberry latent ringspot virus was originally described in 1964, when it was first isolated from strawberry (Lister, 1964). Later, it was isolated

from many other hosts including raspberry (Taylor and Thomas, 1968), celery (Walkey and Mitchell, 1969), *Robinia pseudoacacia* , *Euonymus europaeous* and *Aesculus carnea* (Schmelzer,1969),rose (Cammack,1966 ; Harrison,1967 ; Ikin and Frost, 1976), rhubarb (Tomlinson and Walkey, 1967), *Tussilago farfara* (Taylor and Thomas, 1968), asparagus, blackberry, blackcurrant, redcurrant, cherry, elderberry, grape, plum, peach, rhubarb and narcissus (Murant, 1974).

Strawberry latent ringspot virus can be latent in many hosts, including strawberry, raspberry and blackberry (Lister, 1964). Symptoms induced by SLRV, however, often depends on the host species or cultivar infected. Thus, while SLRV may be symptomless in some strawberry cultivars in cv. Cambridge Vigor it induced chlorotic mottling of foliage and severe stunting of infected plants (Lister, 1964). Similarly, in raspberry cv.Malling Jewel SLRV caused cane stunting and yellow vein banding symptoms, while in blackberry cv. Himalaya Giant SLRV infection resulted in loss of vigour, leaf mottling and small unmarketable fruits (Murant, 1974). Infection in celery cv. Lathom Blanching was associated with severe symptoms known as 'strap leaf' in which leaves were reduced in size and had incurved margins with distorted and stunted petioles and resulted in up to 30% unmarketable crop (Walkey and Mitchell, 1969). In peach SLRV was associated with rosette willow leaf (Ministry of Agriculture, Fisheries and Food, 1974) and in rose with

chlorotic leaf spotting and stunting (Cammack, 1966 ; Harrison, 1967 ; Ikin and Frost, 1976). However, in parsnips SLRV infection was associated with only mild symptoms (chlorotic spots and faint mottling) produced in foliage under colder condition (Cooper, 1981).

Isolates of SLRV from different hosts closely resembled each other in their physical and serological properties, although they differed in virulence (Lister, 1964; Murant, 1974).

Strawberry latent ringspot virus is transmitted by two ectoparasitic nematodes of the genus ***Xiphinema, X. diversicaudatum*** (Lister, 1964 ; Harrison, 1967 ; Taylor and Thomas, 1968) and ***X. coxi*** Tarjan (Putz and Stoky, 1970). ***Xiphinema diversicaudatum*** is most often found in deciduous woodlands or hedgerows (Harrison and Winslow, 1961 ; Pitcher and Jha, 1961 ; Taylor and Brown, 1976 and Thomas, 1970). Adults and larvae both transmit the virus (Murant, 1974). It has been reported that the virus is retained for up to 84 days in ***X. diversicaudatum*** kept without plants (Taylor and Thomas, 1968). Virus-like particles were associated with the cuticular lining of the lumen of the odontophore and the oesophagus of ***X . diversicaudatum*** , fed on plants infected with SLRV (Murant, 1974).

Strawberry latent ringspot virus naturally infects a range of endemic wild plants including many

weeds and is also transmitted through the seeds of a number of hosts (Lister and Murant, 1967). The weeds may act as reservoirs for subsequent spread by previously non-infective nematodes or nematodes rendered non-infective after a period of overwintering fallow ground (Murant and Lister, 1967). Seed transmission of SLRV has been reported in many hosts, where transmission often exceeded 70% (Murant, 1974). More than 10 species, including parsnips, were reported in which SLRV was seed transmitted, namely ***Mentha arvensis*** 6% (Taylor and Thomas, 1968), ***Laminum amplexicaula*** 73%, ***Rubus idaeus*** 75%, ***Stellaria media*** 97% (Murant and Goold, 1969) , ***Chenopodium quinoa*** 63 - 100% (Schmelzer, 1969 ; Allen ***et al .,*** 1970 ; Hicks ***et al.,*** 1986), ***Apium graveolens*** 98 - 100% (Walkey and Whittingham-Jones, 1970), ***Petroselinum crispum*** (Mill) Nym. 7% (Bos ***et al.,*** 1979 ; Harrison and Campbell, 1979), ***Capsella bursa -pastoris*** 4%, ***Senecio vulgaris*** , 20% (Review of App. Mycol. (48) 3247), ***Aesculus carnea*** 74%, ***Euonymus europaeus*** 85% (Schmelzer, 1969) and ***Pastinaca sativa*** 90 - 100 % (Cooper, 1981 ; Hicks ***et al.,*** 1986). The temperature under which infected plants were grown had a great influence on the percentage of infected seed set by a plant. For example, SLRV was found to be most frequently seed-borne in ***Stellaria media*** (chickweed) at a temperature of 22°C (Hanada and Harrison, 1979).

There is evidence that nepoviruses enter the seeds through the pollen as well as through the ovule,

although the percentage seed transmission through pollen is quite low for many nepoviruses (Lister and Murant,1967). An example for this type of spread was with cherry leaf roll virus (CLRV) in birch (Cooper, 1976). Callahan (1957) also reported that elm mosaic virus (now known to be a strain of CLRV) was transmitted 30.5% through pollen and 75% through ovule.

Because of the slow migration of nematodes through soil, the vectors transmission is probably only important in the local spread of the virus. Long distance spread probably occurs by dispersal of infected seeds or possibly by transfer of viruliferous vector nematodes in soil adhering to the roots of planting material (Harrison, 1977; Murant, 1983). Therefore, the seed transmission of SLRV is important in the virus' ecology.

Strawberry latent ringspot virus (Lister, 1964) resembles the nepoviruses in having nematode vectors and polyhedral isometric particles with slightly angular outlines and a diameter of 28 - 30 nm (Cadman, 1963; Harrison ***et al.,*** 1971). It is also similar to nepoviruses in that its genome RNA (single-stranded) consists of two species of relative molecular mass (Mr) 2.9 x 10^6 and 1.4 x 10^6 (Murant ***et al.,*** 1981) which include polyadenylate sequences (Mayo ***et al.,*** 1982) and a genome-linked protein. Strawberry latent ringspot virus has been classified as a tentative nepovirus (Murant,

1981), however, because its coat protein comprises two different polypeptides of estimated Mr 44,000 and 29,000 (Mayo ***et al.,*** 1974; Gallitelli ***et al.,*** 1982) instead of one polypeptide of Mr 52,000 - 58,000 (Mayo ***et al.,*** 1971) for definitive nepoviruses.

Particles of some isolates of several nepoviruses contain RNA species additional to the major two comprising the virus genome. Those found in particles of tobacco ringspot virus have been shown to be 'satellite' RNA because they depend on tobacco ringspot virus for multiplication but are not an essential part of the helper virus genome (Schneider, 1969 ; Murant ***et al.,*** 1973. Furthermore, the satellite RNA does not have extensive sequence homology with genome RNA of the helper virus (Rezaian and Jackson, 1981 ; Robinson, 1982). The additional RNA species found in particles of myrobalan latent ringspot virus, chicory yellow mottle virus and arabis mosaic virus are also likely to be satellite molecules (Murant and Mayo, 1982). A third species of RNA has also been found in SLRV particles of H isolates from strawberry (Mayo ***et al.,*** 1974 ; Gallitelli ***et al.,*** 1982) but not in particles of J isolates (Mayo ***et al.,*** 1974) or in isolates from peach or olive (Gallitelli ***et al.,*** 1982). The third RNA species found in SLRV isolate H is reported to be a 'satellite' RNA with Mr of 0.45×10^6 (Gallitelli ***et al.,*** 1982), or 0.4×10^6 (Mayo ***et al.,*** 1982).

In cells of infected plants SLRV induced the formation of tubular structures containing virus-like particles (Walkey and Webb, 1968 ; 1970 ; Roberts and Harrison, 1970 ; Murant, 1974 ; Ikin and Frost, 1976 ; Babini and Bertaccini, 1982). In positively-stained ultra-thin sections these tubules were double-walled, slightly flexuous about 50 nm wide and up to at least 2.5 µm long. Each tubule (occasionally more than one) was enclosed in a membranous sheath 80 - 120 nm. in diameter, joined to the endoplasmic reticulum (Roberts and Harrison, 1970). Morever,these tubules contained a single row of more than 100 virus-like particles which were all darkly stained with 'positive stains'(Roberts and Harrison, 1970; Babini and Bertaccini, 1982). In negatively stained tissue homogenates and sap extracts, all these virus-like particles remained completely unpenetrated by stain (Walkey and Webb, 1968, 1970; Ikin and Frost, 1976). Hicks (1985) reported that some of the particles in his homogenates of SLRV infected ***Chenopodium quinoa*** leaves were completely penetrated by negative stain. This is common for negatively-stained purified preparations of nepoviruses including SLRV (Murant, 1981).

Although SLRV has been isolated from many natural hosts, it is not always clear from the literature whether it caused the associated symptoms.For example, attempts made by previous workers to infect apparently healthy parsnips with SLRV in accordance with Koch's

Postulates (Koch, 1882) were unsuccessful and parsnips could not be infected by mechanical inoculation (Cooper, 1981) or by grafting techniques and dodder transmisson (Smith,1986). However, Smith (1986) found that when SLRV-infected parsnips cv. White Gem were grafted to healthy parsnips, a single plant of cv. Tender and True became symptomlessly infected seven weeks after grafting. Similarly, Cammack (1966) was also unsuccessful in inoculating SLRV to rose by sap inoculation although a few rose plants became infected with SLRV by graft-inoculations.

The objectives of the present work were to characterise SLRV isolates from parsnip with respect to biological, physical and chemical properties. Different isolates of SLRV from parsnips cv. White Gem were compared with each other, and with an isolate from the woody ornamental ***Caryopteris clandonensis***. Two isolates were purified and antisera produced.

Only a little information is available on the effects of SLRV infection on parsnip yield. Hicks ***et al.,*** (1986) reported that the quality of parsnip seeds from mother plants that were infected with SLRV were reduced with respect to their seed weight and percentage germination. This work was extended to determine the effects of SLRV infection on the root yield of parsnip and also the effects of infection on experimental hosts with

respect to seed production, germination and seed quality.

Seed transmission of SLRV isolates from parsnip was compared for different isolates. Factors affecting seed transmission of SLRV were studied and attempts made to inactivate or eradicate virus from seed.The role of pollen in transmission of SLRV was also examined.

Attempts were made to inoculate SLRV isolates, to healthy parsnip plant, to demostrate Koch's Postulates,as defined by Bos for plant viruses (Bos,1983).

Parsnip yellow fleck virus (PYFV)

Like SLRV, PYFV is also confined to Europe and is found naturally in Great Britain (Murant and Goold, 1968) in Germany (Wolf and Schmelzer, 1972) and in the Netherlands (Dijk and Bos, 1985). The first report of PYFV dates back to 1968, when Murant and Goold (1968)isolated it from parsnip plants with virus-like symptoms from various parts of England and Scotland. The virus was apparently similar to a previously isolated virus from symptomless plants of cow parsley (Murant and Goold, 1968). Murant and Goold (1968) concluded that PYFV was the commonest cause of virus-like symptoms in parsnips in Great Britain.

Apart from parsnip (***Pastinaca sativa*** L.) and cow parsley (***Anrthriscus sylvestris***), both in the

family Umbelliferae, the virus has been isolated from many other umbelliferous hosts including hogweed (*Heracleum spondylium* L., Tomlinson and Carter, 1970; Bem and Murant, 1979; El Nager and Murant 1974; Waterhouse and Murant,1981 and Dijk and Bos, 1985), celery (*Apium graveolens* var dulce, Singh and Frost, 1987),carrot (*Daucus carota* L., Waterhouse and Murant,1981; Dijk and Bos,1985), *Anethum graveolens* (dill), *Anthriscus crerfolium* (chervil), *Coriandrum sativum* (coriander), *Chaerophyllum temulum, Oenanth aquatica, Trlllis japonica* and *Aethusa cynapium* (Dijk and Bos,1985). Parsnip yellow fleck virus has a rather limited host range, systemically infecting 11 out of 19 umbellifererous plants and 12 out of 23 species in eight other families (Murant and Goold,1968 ; Hemida and Murant, 1989). A further fifteen non-umbelliferous plants were infected in inoculated leaves only (Murant and Goold, 1968). Natural infection of PYFV has only been reported from umbelliferous plants.

Parsnip yellow fleck virus isolates could be divided into groups: those from parsnip, celery and hogweed belonged to P-121 serotype (parsnip strains) while those from carrot and cow parsley belonged to A-421 (*Anthriscus strains*) serotype (Hemida and Murant, 1989). In addition to this natural host separation, isolates of the two serotypes differed in their experimental host ranges. For example, Dijk *et al.*, (1987) reported that five *Nicotiana* species namely, *N. linestris* , *N.*

hesperis, N. occidentalis , N. megalosiphon and *N. suaveolens* are hosts of isolate P-121 but not of isolate A-421. Similarly Hemida and Murant (1989) reported that isolates of A-421 serotypes differed from those of P-121 in not infecting *Datura stramonium N. glutinosa* and *Physalis floridana.* In addition, there are minor variations in serological properties, host range and symptom expression among isolates within each serotype (Hemida and Murant, 1989).

Symptom expression in plants mechanically inoculated with PYFV, in general, varied from mild local chlorotic spots to severe systemic necrosis, distortion die-back and eventually plant death (Murant and Goold,1968; Dijk and Bos,1985; and Hemida and Murant,1989), depending to some extent on the hosts and the serotypes. Thus, strains of A-421 serotype were unable to induce systemic reaction in *N.clevelandii* whereas P-121 strains produced a prominent systemic chlorotic mottle in this host (Murant and Goold,1968).

Parsnip plants naturally infected with PYFV showed vein yellowing, yellow flecks on leaves or a more definite yellow and green mosaic. Plants were somewhat stunted but were not normally killed by the infection (Murant and Goold, 1968). Celery infected with CV 506 (celery isolate of PYFV) showed yellow netting, systemic chlorosis and crinkling of leaves, stunting and

sometimes death (Singh and Frost, 1987). Infection in carrot ranged from mild mottle and occasional reddening, to rapid dieback and plant death, although the tap root appeared normal (Dijk and Bos, 1985). During 1979 - 1984 disease incidence in carrot seed crops on the island of Tholen (in the south west of the Netherlands) varied from 10% - 80% and the disease appeared to be especially damaging the seed plants in the carrot breeding programme (Dijk and Bos, 1985). In contrast, infection in cow parsley was latent (Dijk and Bos,1985).

Parsnip yellow fleck virus is known to be transmitted in the semi-persistent manner by the aphid ***Caveriella aegopodii*** scop., and by ***C. pastinace*** (Murant, and Goold,1968).Transmission by ***C. aegopodii*** depended on the presence of a helper virus in the source plant, ***Anthriscus*** yellows (AYV, Murant and Goold, 1968). The aphids retained both PYFV and AYV up to 4 days, but decreased gradually on feeding and lost the viruses on moulting. ***Anthriscus*** yellows virus is also semi-persistent in the vector (Elnager and Murant, 1973), but is not transmissible by inoculation of sap (Murant and Goold,1968). Parsnip yellow fleck virus is not normally transmitted by aphids from manually inoculated plants nor from plants immune to AYV including parsnip and carrot. However, aphids already carrying AYV can acquire PYFV from singly-infected plants or feeding through membranes on plant extracts of purified virus preparations (Elnager and

Murant, 1973).

In Britain, hogweed (*Heracleum spondylium*) is reported to be the main source of PYFV in which AYV could also occur (Bem and Murant, Tomlinson and Carter, 1970). Dijk and Bos (1985) have reported that in the Netherlands, PYFV was prevalant in hogweed and in cow parsley where the latter species was also a host of AYV. The latent infection of PYFV in cow parsley and the perennial nature of the host explain the high incidence of infection with PYFV and helper virus AYV.

Particles of PYFV are isometric, 29-30 nm in diameter (Murant and Goold, 1968), with a single ssRNA genome of Mr about 3.7×10^6 (Murant ***et al.,*** 1974) or 3.4×10^6 (Hemida and Murant, 1989). Viral coat protein comprises three polypeptides with estimated relative molecular masses (Mr) of 30,27 and 22 ($\times 10^{-3}$) (Murant, ***et al.,*** 1974) or of 30, 26, 24 ($\times 10^{-3}$) for A-421 and 31, 26 and 23 for P1-121 (Hemida and Murant, 1989). Moreover, the peptide maps for the three proteins of an isolate were different indicating that the proteins were different and not derived one from another (Hemida, ***et al.,*** 1985). The medium-sized proteins of the two PYFV strains were reported by the same authors to be least similar by peptide mapping, but most similar serologically.

Parsnip yellow fleck virus induced the formation of inclusion bodies in leaf cells of infected

spinach, ***N. clevelandii*** and ***Anthriscus cerefolium***. Inclusions occurred in most types of cell often within vesicles, (especially in mature inclusion bodies,) and consisted of straight tubules c.30nm in diameter (Murant, ***et al.,*** 1973). Infected cells also possessed plasmodesmatal and cytoplasmic tubules c. 45nm in diameter containing virus particles ; plasmodesmatal tubes were sheathed by cell wall outgrowths (Murant, ***et al.,*** 1973).

The properties of particles suggest that it should be regarded as the type member of a new taxonomic group of plant viruses. In the list of viruses known to be dependent on others for transmission by aphids (Rochow,1972), PYFV is the only one that is transmitted in the semi-persistent manner. For example, tobacco mottle virus (Smith,1946) and carrot mottle virus (Watson ***et al.,*** 1964) are both persistent whereas potato virus C (Watson, 1960) and potato aucuba mosaic virus (Kassanis, 1962) are non-persistent. The isometric viruses that may be regarded as semi-persistent, constitute a heterogeneous group in particle morphology. For example, cauliflower mosaic and related viruses have 50 nm particles and black raspberry virus (Matthews, 1981) and AYV (Murant and Roberts, 1977) have 29 - 30 nm particles.

The particle properties of AYV and dandelion yellow mosaic virus (DYMV, Kassanis,1944,1947 ; Bos ***et al.,*** 1983 ; Vetten ***et al*** .,1985) showed a

similarity to PYFV (Hemida *et al* ., 1986). *Anthriscus* yellows virus is similar to PYFV in being a semi-persistent virus transmitted by the aphid *C. aegopodii* (Elnager and Murant, 1976 a) and in having isometric particles 29 nm in diameter (Murant and Roberts, 1977) with a single species of ssRNA, of Mr 3.6 x 10^6. Moreover, particles contained four proteins, 35,28,24 and 22 (x10^{-3}) (Hemida *et al.,* 1989). Anthriscus yellows virus and PYFV somewhat resembled picornaviruses of vertebrates and insects in shape, size and RNA composition (Hemida *et al.,* 1986)

Unlike PYFV, however, AYV was not mechanically transmitted and was confined to phloem tissues (Elnagar and Murant,1976) whereas PYFV occurred in most type of cells. *Anthriscus* yellows virus induced the formation of 'currant bun' inclusion bodies,in which the virus particles were embedded in a proteinaceous matrix (Murant and Roberts, 1977). The two viruses were not serologically related (Hemida *et al.*, 1989).

The affinity of PYFV to DYMV is strong (Hemida *et al.*,1986). Dandelion yellow mosaic virus was both aphid and sap transmissible, had isometric particles of 30nm in diameter and a single polyadenylate RNA species of Mr 3.3 x 10^6 (Hemida *et al.,* 1986). Moreover, it contained three protein species of estimated Mr of 32,29 and 26 (x10^{-3}) where the polypeptide maps were different from each other(Hemida *et al.,* 1986). However, DYMV was

not related to PYFV serologically. The PYFV, therefore, can be regarded as the type member of a new taxonomic group of isometric plant viruses (Hemida and Murant,1989).

In addition to work on SLRV an isolate of PYFV from parsnip cv. White Gem was characterised with respect to its biological, physical and serological properties. The parsnip isolate and an isolate of PYFV from *Angelica archangelica* were purified and antisera were produced. An investigation was also made of the interaction of PYFV with SLRV , and of seed transmission of PYFV, using ELISA techniques.

CHAPTER 2 Materials and Methods

2.1 Source of infected plants

In an initial survey parsnip cv. White Gem plants about one year old, with virus-like symptoms, growing at the Bath University Field Station, were tested for virus. A proportion of infected plants were lifted from the field and potted into 30 cm pots and grown in a glasshouse with a temperature range 17 - 34°C. Apart from field plants, virus isolates were also obtained from batches of commercial seed (Suttons and Unwins Seed Ltd.).

2.2 Culture of test plants.

(i) Source of seeds

Parsnip seeds and seeds of most herbaceous test plants were collected from apparently virus-free mother plants which were raised in the glasshouse at Bath for seed production. Seeds from commercial packets were also used, obtained from following sources:

Amaranthaceae : ***Gomphrena globosa*** L.
(globe amaranth).
Suttons Seeds Ltd.

Cucurbitaceae : ***Cucumis sativus*** L
cvs. Parisiene pickling,
Sharpes and Co. PLC
Marketer
Butchers Disease Resister,
Ridge Crystal Apple
Suttons Seeds Ltd.

Leguminoseae : ***Phaseolus vulgaris*** L.(french bean)
Sharpes and Co. PLC
Vicia faba L.
Sutton and Son Ltd, Reading.
Phaseolus multiflourus(runner bean)

Umbelliferae : *Apium graveolens* L. (celery)
cv. Solid White
Anethum graveolens L.(dill)
Coriandrum sativum L.(coriander)
Pastinaca sativa L. (parsnip)
cvs. Hollow Crown
Tender and True
Sutton Seeds Ltd.
White Gem
Unwins Seeds Ltd, Sutton Seeds Ltd
Daucus carota L. (carrot)
cv. Royal sluis
Sanokote Enkhuizen-Holland
Anthriscus cerefolium (chervil)
Lifepak

Chenopodiaceae : *Beta vulgaris* L. (beetroot)
cv. Globe - Sutton Seed Ltd.
Spinacea oleracea L.(spinach)
Lifepak

(ii) Culture of test plants

Seeds of test plants were sown in trays of Universal Levington Compost and germinated in a mist-house at 21-24°C. When the seedlings were an appropriate size to handle, they were pricked into 10 cm pots in Levington potting compost and were grown in an insect-protected glasshouse at 17 - 34°C.

2.3 Inoculation procedures

Plants to be inoculated were kept in a dark box for at least 24 h prior to inoculation, as this has been found to increase susceptibility to infection (Bawden and Roberts, 1948). For host range studies young leaves with clear symptoms were homogenised in cold, 0.05 M potassium phosphate buffer at pH 7.8 (1 : 1 w / v, unless otherwise stated), in a chilled mortar and pestle

containing a small amount of 100-mesh carborundum to aid grinding. A little celite abrasive was finally added to the crude sap extract and plants were inoculated by lightly rubbing sap onto the leaves with a finger. The leaves were then rinsed briefly with tap water, (Holmes, 1929 ; Yarwood, 1973).

After inoculation, plants were covered with a single sheet of damp newspaper for about 24 h to provide a humid environment and protect from light and wilting. Plants were then grown in a glasshouse with a temperature range of 17-34°C. During the summer months the glasshouse was shaded with coolhouse white wash (PBI Ltd.) to reduce incident sunlight and temperature. In the winter a supplementary lighting from mercury vapour lamps (3,500 - 4,000 lux) was given. For some experiments, plants were grown in growth cabinets with a photoperiod of about 7,000 lux illumination at temperatures of 18° and 26 ± 1°C.

2.4 Establishment of single lesion isolates

Each virus was established as a single lesion isolate to eliminate the possibility of virus mixtures. Single lesions were cut out and ground in a pre-chilled solid watch glass with a test tube in a drop or two of inoculation buffer. After adding a little celite the homogenate was inoculated to a suitable host seedling. Resulting lesions were passed through 3 - 4 successive transfers and the final cultures were then bulked up

either in the same host or in a suitable propagation host for the virus. All possible steps were taken to prevent contamination of this 'pure' culture during maintenance.

2.5 Quantitative experiments

When infectivity was assessed by local lesion counts, half leaves of test plants were (*Phaseolus vulgaris*) inoculated in balance incomplete block design or, with cucumber, 'both cotyledons'. Lesion numbers x were transformed to values Y (to normalise the data) according to the following formulae (Kleczkowski, 1968)

$$Y = \log_{10} (x + c), \quad \text{for } x > 10$$

$$Y = \log_{10} \; 1/2 \{ x + c / (x^2 + 2cx)^{1/2} \} \text{ for } 1.5 < x < 10,$$

where c is a constant

Results were analysed using Student's t-test. Other data were subjected to analysis of variance or chi-squared tests (Snedecor and Cochran,1967). In the case of germination percentages, the data were first transformed to angles and analysed by chi-squared test.

2.6 Pollen and seed transmission

Seeds were collected from many infected host plants which had been manually inoculated at young stages with SLRV, and grown at a temperature range of 17-34°C. Parsnip seeds were collected from naturally-infected mother plants. Seed transmissibility was determined either

by inoculating buffered seed extracts onto *C. quinoa* seedlings or by sowing the seeds for germination and then indexing the seedlings on *C.quinoa*. Some seedlings were also tested by ELISA (2.12). Pollen from infected and disease-free plants was collected with a small paint brush into a watch glass and macerated in a drop of inoculation buffer and inoculated to *C. quinoa* .

In vitro germination of pollen

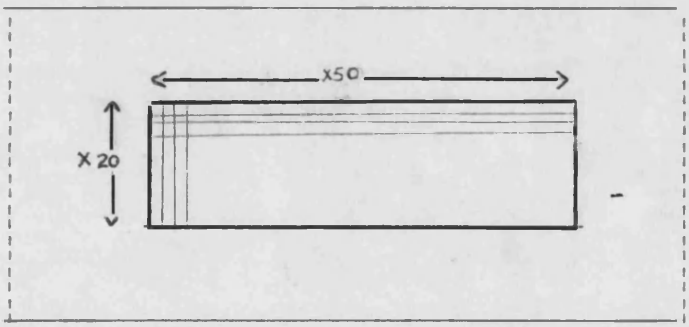
A method used by Yang and Hamilton (1974) was followed with a slight modification to study pollen production and *in vitro* germination of pollen from SLRV infected plants and 'healthy' plants. To study pollen production, immature anthers were collected (just before dehiscence) to ensure that all pollen grains to be counted came from one anther, and macerated with a glass rod in a small test tube containing a small volume of water. The pollen suspension was diluted with 50% glycerol (which helps to deposit pollen grains evenly in the counting chamber) and thoroughly mixed before the samples were taken for counts.

To study *in vitro* germination, pollen was collected either from already opened anthers or from anthers before dehiscence and macerated as above, and allowed to germinate in medium containing 25% sucrose and 100 ppm boron (H_3BO_3). Samples were examined under a light microscope in 3 - 4 h or after allowing them to germinate

overnight.

Counting of pollen

One ml of the pollen suspension was layered into a counting chamber which has a well of 1 ml capacity divided into 1000 square wells; thus, each well holds 1 µl of the suspension. A cover slip was then carefully placed over the well and the number of pollen grains counted in randomly selected wells (about 8-12 wells), under a light microscope using the lower magnification (4x10). A diagrammatic illustration of the counting chamber is shown below:



2.7 Nematode transmission experiments

2.7a Isolation of nematodes from soil

Soil samples were collected from the University ground where several rosaceous shrubs had been growing for the previous 10 years. Samples were taken by a shovel to depth of about 15 cm and the nematodes, ***Xiphinema diversicaudatum*** (Micoletzky) were extracted from 200 g subsamples by wet-sceening on 2 mm and 124 µ

sieves (Taylor and Thomas, 1968). Each 200 g soil sample was broken up with 800 ml tap water and allowed to stand for half an hour while mixing thoroughly but gently with a glass rod, from time to time. The soil suspension was then washed through a 2 mm aperture sieve to remove large stones and debris. Tap water was then added to the resulting suspension to about five litres, which was gently stirred and allowed to settle for 20 sec. It was then decanted through a 124 μ aperture sieve, and the residue on the sieve washed with tap water from a wash bottle and collected into a beaker. Samples from the beaker were examined through a dissecting microscope (x10), and nematodes were picked carefully by means of a pasteur-pipette and examined under a light microscope for identification.

2.7b Nematode inoculation

The infectivity of the nematode population in these soils was assessed by mixing the soil thoroughly and growing 'bait' plants for 6 weeks in samples contained in 10 cm pots (Cadman and Harrison, 1960). Sap of both leaves and roots from bait plants was indexed on ***C.quinoa***. The bait plants were cucumber (***Cucumis sativus*** L. cv. Marketer), pea (***Pisum sativum*** L. cv. Meteor) and beetroot (***Beta vulgaris*** L. cv. Globe).

In virus transmission experiments, initially non-infective nematodes were handled as

described by Harrison and Cadman, (1959) and added to 10 cm pots containing potting compost in which 4 - 5 SLRV-infected parsnip or other test seedlings were grown. After allowing the nematodes to feed for 5 - 6 weeks, infected parsnips/test plants were removed and soil sieved through a 6 mm sieve to remove any large root fragments. Healthy parsnip or *C. quinoa* test seedlings were then introduced to these soils in 10 cm pots, and the incidence of infection by SLRV determined by indexing sap from roots and leaves of these seedlings on *C. quinoa*.

In later experiments, both the source plants and plants to be inoculated were directly potted in the soil containing nematodes which was treated with fertilizer (balanced N P K) when necessary.

2.8 Graft transmission experiments

Inverted saddle graft

Detached-scion grafting technique was used to graft parsnip roots. Equal-sized 'scions'(healthy) and 'rootstocks' (SLRV-infected) were chosen to permit and facilitate matching of the two cambial layers. An inverted saddle graft (Mahlstede and Haber, 1957) was done making two equal diagonal cuts on either side of the central pith area in an downward direction in the 'rootstock', (in this case, infected parsnip) and the resulting central piece was removed. Diagonal cuts of equal length were then made on the 'scion'(healthy parsnip) in the form of a saddle to

match the cut in the rootstock. The two components were then fitted together and tied securely in place with strips of parafilm and potted in Levington compost in 20 cm tall pots.

2.9 *In vitro* properties

Tests were carried out to determine the thermal inactivation point (TIP), dilution end point (DEP) and longevity ***in vitro*** (LIV), following standard procedures (Bos ***et al.,*** 1960 ; Noordam, 1973). Although these tests are now not considered to be of great diagnostic value (Hamilton ***et al.***, 1981) they are still quite valuable in giving an indication of stability and concentration of a virus and, thus, are useful in developing a purification method.

The source of inoculum used to determine these physical properties was ***C. quinoa*** leaves recently infected with SLRV isolates. In the case of PYFV systemically-infected ***N. clevelandii*** leaves were used. The leaf tissue was ground in cold phosphate inoculation buffer (1 : 0.5 / w : v) at pH 7.8 and the mixture filtered through muslin.

The thermal inactivation point (TIP) is the temperature that completely inactivates the virus during a 10 min exposure. Aliquots of infective sap in test tubes were heated at desired temperatures (range of 50 - 70°C) in a dry block apparatus. Care was taken not to

contaminate the wall of the test tubes when sap was added to the tube for heating. After 10 min the tubes were cooled immediately, and the infectivity of the sap was assayed by inoculation to suitable hosts.

The dilution end-point (DEP) of a virus is generally stated as between two dilutions ie. between the highest dilution that is still infective and the next highest one. Five or ten fold dilutions of the infective sap were made in inoculation buffer, and the dilutions assayed by inoculation to young test hosts.

Longevity ***in vitro*** (LIV) is defined as the time that virus in crude sap kept at room temperature remains infective. This test was extended to include crude sap kept at 4°C. Aliquots of infective sap were maintained at these temperatures in stoppered bottles in the dark, and the infectivity of the sap determined at desired intervals by inoculation to suitable host plants.

No satisfactory local lesion host for SLRV isolates was found and, therefore, in this study the virus concentration was assayed by determining the ID_{50} (infective dosage giving 50% infected plants) based on the number of systemically-infected ***C.quinoa*** (Read and Muench, 1938). In the case of PYFV, a local lesion host-***N. tabacum*** cv. White Burley was used and at least five plants selected for uniformity were inoculated on four fully expanded leaves with each treatment.

2.10 Purification of virus

Isolates of SLRV for purification were cultured in ***C. quinoa*** and for PYFV in ***N. clevelandii*** . The schemes used were based either on the method of Fulton (1959) using hydrated calcium phosphate (HCP) followed by concentration of virus with polyethylene glycol (PEG) Mr 6,000 (Hebert, 1963) or on the method of Mayo ***et al.,*** (1982) using n-butanol clarification followed by PEG treatment.

(i) Method based on HCP clarification

Leaves and petioles of ***C. quinoa*** recently infected with SLRV harvested 8 - 10 days after inoculation (unless otherwise stated) were homogenised in a wareing mechanical blender with HCP and 0.05 M phosphate buffer pH 7.8 (extraction buffer) plus 5% Polyclar AT and 0.01M sodium thioglycolate (1g tissue of leaves : 1 ml extraction buffer : 0.6 g HCP). Hydrated calcium phosphate was prepared according to Fulton (1959).

Once the leaf extract with HCP appeared homogeneous, the sap was expressed through muslin and clarified as follows (all steps at 4°C):

(i) centrifuged at 5,000 g for 20 min and pellet discarded.

(ii) 8% PEG and 0.03 M NaCl were added to the supernatant and stirred for 1-2 h

(iii) centrifuged at 5,000 g for 20 min and the supernatant discarded.

(iv) pellet resuspended in 0.01 M phosphate buffer pH 7.8 (suspension buffer) overnight

(v) centrifuged at 800 g for 30 min and pellet discarded

(vi) supernatant diluted in suspension buffer and centrifuged at 125,000 g for 2 h

(vii) pellets resuspended in suspension buffer overnight

(viii) centrifuged at 800 g for 10 min and pellet discarded

The supernatant containing partially-purified virus was stored at -20°C until required.

Alternatively, a method used by Mayo ***et al.,*** (1982) was followed to purify SLRV from infected leaves of ***C. quinoa.***

Infected leaf materials were homogenised in 0.07 M phosphate buffer, pH 7 plus 0.1% 2-mercaptoethanol and 0.01M ethylenediamene tetra-acetic acid (EDTA) at a rate of 1:1/w:v. The sap was filtered and clarified as follows (all steps at 4°C)

(i) 8.5% n-butanol was added to the supernatant and stirred for 1-2 h

(ii) centrifuged at 5,000 g for 20 min and pellet discarded

(iii) 10% PEG and 1% NaCl were added to the supernatant and stirred for 2h

(iv) centrifuged at 5,000 g for 20 min and supernatant discarded

(v) pellet resuspended in 0.017 M phosphate buffer pH 7 (suspension buffer) overnight

(vi) centrifuged at 800 g for 30 min and pellet discarded

(vii) supernatant centrifuged at 125,000 g for 2h.

(viii) pellets resuspended in suspension buffer overnight

(ix) centrifuged at 800 g for 10 min and pellet discarded

Partially-purified virus was stored at -20°C until required.

Purification of PYFV was done by n-butanol clarification using the same extraction buffer and suspension buffer as described for SLRV purification.

Further purification of partially-purified virus preparations was achieved by permeation chromatography (Halter, 1965, 1967) on columns of controlled pore glass (CPG, Barton,1977) which acts as a 'molecular sieve'.

Dry CPG of mean pore diameter 72 μ suspended in 0.01 M degassed phosphate buffer pH 7.8 was loaded onto the chromatography column (Whatmans 1.5 cm diameter) as described by Barton (1977). After the column had equilibriated in 0.01M phosphate buffer about 0.5-1.0 ml of partially purified SLRV samples were applied and buffer eluted at a rate of 15 ml / h at room temperature; the eluates were monitored at 254nm with a LKB Uvicord S analyser.

Functions from individual peaks were pooled and centrifuged at 125,000 g for 2 h. The pellets were

then dissolved in 0.01 M phosphate buffer (in the case of n-butanol clarification, the suspension buffer was 0.017 M phosphate buffer at pH 7) overnight and then centrifuged at low speed for 10 min.

The absorption spectra of the purified samples (at various stages) was measured and the purity and the concentration of the virus preparation determined using a Shimadzu UV scanning spectrophotometer. The virus concentration was estimated using the following equation:

$$\text{concentration of virus (mg/ml)} = \frac{\text{Absorbance at 260nm}}{\text{Extinction coefficient}} \times \text{dilution factor}$$

An assumed extinction coefficient of 7 was used for purified SLRV particles based on that for a typical nepovirus (Mayo ***et al.,*** 1974)

2.11 Serology

2.11a Production of antiserum

Highly purified virus preparation were used to produce antisera for several isolates of SLRV. For PYFV,a partially-purified preparation (after two cycles of high and low speed centrifugation) was used and the antiserum cross absorbed against partially-purified preparations from healthy ***N. clevelandii.***

Rabbits (Sandy half lop) were injected in

the ear intravenously with 0.5 ml of virus suspension, diluted in saline (0.85% NaCl) to give a concentration of about 0.5 mg/ml. Another three intramuscular injections were given at weekly intervals after the first intravenous injection, mixed with an equal volume of Freund's incomplete adjuvant (Difco Labs).

Rabbits were bled six times at weekly intervals starting the third week after the first injection. Blood samples were left to coagulate in sealed tubes at room temperature for 4h and kept at 4°C overnight. Clear serum containing antibodies was carefully collected with a Pasteur pipette and stored at -20°C in 50% glycerol.

Antisera to type strain SLRV and to AMV (*Arabis* mosaic virus) was kindly supplied by Dr.A.F.Murant (Scottish Horticultural Research Institute, Invergowrie, Dundee) while antiserum to PYFV was kindly supplied by Dr.D.G.A.Walkey (Institute of Horticultural Research, Wellesbourne).

2.11b Gel double-diffusion test

The Ouchterlony gel double-diffusion procedure (Ouchterlony,1962 ; Crowle, 1973) was used for serological tests using a Gelman immunodiffusion equipment. Six microscope slides were placed on an immunoframe and their surfaces and cracks between them sealed with a small

amount of molten agar,prepared by dissolving 7.5g Oxoid ion Agar No. 2 , 7.5 g NaCl and 0.4 g sodium azide in 1 litre of 0.01 M potassium phosphate buffer, pH 7. Slides were then air-dried and molten agar pipetted onto the slides at a rate about 4 ml/slide, and allowed to air-dry for an hour. Each slide was stamped with desired well patterns using a Gelman punch and the agar plugs removed.

Crude sap was prepared by homogenising infected or uninfected leaf tissues, 1 g in two drops of saline with a sterile pre-chilled pestle and mortar and centrifuging at low speed in a microfuge for 10min at 4°C. The supernatant of these samples or partially and highly-purified virus preparations, were placed in the outer wells, and the central wells filled with a known antiserum. The slides were incubated in a moist chamber at room temperature overnight, and then placed in a refrigerator at 4°C.

2.11c Immunoelectrophoresis

A Gelman electrophoresis apparatus was used for agar-electrophoresis. Agar was made essentially as in 2.11b, except that tris-phosphate buffer (0.08 M Tris, 0.002 M EDTA , 1.5 ml of 85% phosphoric acid) was used, unless otherwise stated at the required pH.

A pattern consisting of troughs and wells was punched out as required using a Gelman punch. Agar was

carefully removed from the wells and purified virus samples were placed into them. Each side of the Deluxe Electrophoresis Chamber was filled with tris-phosphate buffer, of 450 ml. Immunoframes were then placed across the support bridges of the chamber which were placed in outer slots. Microporous wicks saturated in buffer solution were placed on the agar layer (4 per frame) with the opposite end dipping into the buffer solution. Electrophoresis was at 5 mA per frame for 2-3 h at room temperature.

After the run, the gels from the trough were removed with a gel knife and about 200µl of antiserum spread evenly into the troughs; slides were incubated 24-48 h in a humidity chamber at room temperature. The distance moved (mean distance of the leading and trailing edges of the precipitin lines) from the centre of the original well was then measured (Hollings and Stone, 1975).

Gel slides from both double-diffusion and immunoelectrophoresis were stained when necessary, with 0.01M DOPA (3-4 di-hydroxyphenyl-DL alanine, Walkey, 1985).

2.12 Enzyme-linked immunosorbent assay (ELISA)

Numerous procedures of ELISA are now being used in plant virus detection. Of these the double antibody sandwich, a direct ELISA method (Clark and

Adams, 1977), and an indirect method, using a protein A-enzyme conjugate (Lommel *et al.*, 1982) were followed in this study. The buffers and test samples were made as follows.

Phosphate buffered saline (PBS) -0.01 M phosphate (Na_2HPO_4/KH_2PO_4) containing 0.15 M sodium chloride and 0.003 M potassium chloride, pH 7.4; PBS with 0.5 ml Tween 20 (Sigma Ltd.); coating buffer - 0.05 M sodium carbonate, pH 9.6; conjugate buffer - PBS-Tween containing 20 g/l PVP (Mr 40,000, Sigma Ltd.) and 2 g/l ovalbumin (Sigma grade v); substrate buffer -97 ml/l diethanolamine adjusted to pH 9.8 with M hydrochloric acid.

Gamma-globulin was prepared from 2 ml samples of crude antiserum by ammonium sulfate precipitation. The precipitate was resuspended in 2 ml of half-strength PBS and dialysed against three changes of this buffer overnight to remove ammonium sulphate. This gamma-globulin preparation was further purified by passing through a column of DE 22 cellulose (Whatman Ltd.) housed in 10 ml burette, to remove albumins. Gamma-globulin was washed from the column with half strength PBS, the 1 ml eluates being monitored at 280 nm and the first protein peak collected. It was then diluted to read OD_{280} 1.4 (concentration of about 1 mg/ml) and stored at -20°C after adding 0.2% sodium azide.

For conjugation with alkaline phosphatase (Sigma Type V11), approximately 1 mg of enzyme precipitate was collected by centrifugation for 10 min at 6,000 g and dissolved in 0.5 ml gamma-globulin solution. After dialysing three times against PBS, 0.6 µl/ml fresh gluteraldehyde (Sigma, electron microscope grade) was added and incubated for 4 h at room temperature. Gluteraldehyde was removed by dialysis against PBS containing 0.2% sodium azide, and the conjugate was stored at -20°C after adding 5 mg/ml bovine serum albumin (Sigma Ltd.)

ELISA was conducted in polystyrene immunoassay microplates (Alpha Laboratories). Gamma-globulin was diluted in coating buffer as required and 200 µl was added to each well with a micropipette. The plate was covered to prevent evaporation and incubated at 30°C for 2 - 4h. After incubation the plate was emptied and washed three times with PBS-Tween, 3 min each time. Care was taken to avoid cross contamination of wells during washings.

Aliquots of 200 µl of test sample made by grinding fresh or frozen infected tissue (leaf, root or whole seedlings) in PBS-TPO were added to duplicate wells. The plates were incubated overnight at 4°C. Healthy control and extraction buffer controls also were applied in duplicate wells.

Plates were washed as before and blot dried. Aliquots of 200 µl conjugate diluted in PBS-TPO were then added to each well and incubated at 30°C for 3 - 6 h. After incubation the plates were given three standard washes and finally 200 µl of freshly prepared substrate, p-nitrophenyl phosphate (Sigma Ltd.) at 1 mg/ml was added and the plate incubated at room temperature, normally for 30-45 min. When necessary, the reaction was stopped by addition of 50 µl 3M sodium hydroxide to each well with thorough mixing.

Quantitative measurements of hydrolyzed enzyme substrate were taken using a Titertek Multiskan plate reader (MR 600 multiplate reader, Dynatech Labortaries, INC). Absorbance at 410 nm (A_{410}) was measured after the plate had been blanked against substrate control wells. Values of A_{410} greater than twice those of healthy controls were considered as positive for virus (Thomas, 1980; Hill, 1984).

When indexing a large numbers of samples by DAS-ELISA, seeds or seedlings were tested in groups of two,four or five and the proportion of plants infected were estimated according to Clark and Bar-Joseph (1984) as follows:

$$L = 100 \left(1 - \sqrt[n]{1-G/100} \right)$$

L, proportion of infected plants
n, number of individuals tested in a group
G, percentage group infected with at least one infected individual

The same buffers and reagents were used for Protein-A ELISA but the plates were not pre-coated with antibodies. The steps were as follows:

(i) test samples extracted either in carbonate buffer (0.01 M Na_2CO_3,0.03 M $NaHCO_3$ containing 0.02% NaN_3) pH 9.6 or in PBS-TPO were added to duplicate well and incubated at 30°C for 2h.

(ii) crude antiserum diluted in PBS-TPO was added and incubated at 4°C overnight

(iii) Protein-A conjugated with alkaline phosphatase (5 mg in 1250 ml PBS-TPO plus 50% glycerol) was diluted 1/1250 in PBS-TPO added to each well, and incubated at 30°C for 4h

(iv) substrate added as for DAS-ELISA and plate developed at room temperature for 5 - 6 h followed by 24 - 48 h at 4°C when necessary.

2.13 Extraction and analysis of viral double-stranded ribonucleic acid (dsRNA)

Analysis of viral dsRNA can be a valuable diagnostic tool, and has several advantages over the other conventional methods of virus detection.

The replication of viral RNA proceeds via the production of a template negative strand RNA (Morris and Dodds, 1979) which can be isolated from plants as replicative form dsRNA. The number, size and the pattern of genomic dsRNA (major ones) and sub-genomic dsRNA (smaller species of dsRNA) are diagnostic for a virus or a virus group, and may be detected by gel electrophoresis.

In this study, dsRNA was isolated following a method described by Dodds *et al.,* (1983). Fresh or frozen infected leaf material was powdered in a pre-chilled mortar with liquid nitrogen. One volume of this powder was then extracted in one volume double-strength STE buffer (single strength -0.1 M NaCl, 0.05 M Tris, 0.001 M EDTA pH 6.8) containing 0.1% 2-mercaptoethanol and 1% sodium dodecyl sulphate (SDS), one volume of water-saturated phenol (phenol : distilled water/ 90 : 100 ;w/v) and 0.5 volume of chloroform pentanol (25:1). The mixture was shaken for 20 min either at 4°C or at room temperature and clarified by centrifugation for 15 min at 8,000 g at 4°C. The supernatant containing nucleic acids in STE buffer was adjusted to 16.5% ethanol (final concentration) and the dsRNA extracted by column chromatography using CF-11 cellulose pre-washed in 16.5% ethanol-STE buffer. The column was thoroughly washed with 16.5% ethanol-STE buffer. The dsRNA was eluted from the columns with single strength STE buffer and precipitated with 2.5 volume of 95% ethanol and 1 / 20 volume of 3 M sodium acetate, and stored at -20°C.

The dsRNA pellets were collected by centrifuging at low speed (800g) for 30 min and dissolved in 100 µl electrophoresis buffer (1 mM EDTA,40 mM Tris, 20 mM sodium acetate, at pH 7.8) containing 15% glycerol.

Analysis of dsRNA by polyacrylamide gel electrophoresis

The dsRNA was electrophoresed in 6% polyacrylamide tube gels (7.5 cm x 6mm) prepared by mixing the following ingredients.

4.0 ml of acrylamide (15%) + bis (0.375%) mixture
3.3 ml of 3 x electrophoresis buffer
4.9 ml of distilled water
0.2 ml of 10% fresh ammonium persulfate
0.02 ml of TEMED (N',N',N',N' tetramethyl ethylenediamine)

Lambda DNA cut with EcoR 1 and Hind 111 restriction enzymes (Boehringer Corporation Ltd.) were used as molecular weight markers. The dsRNA samples of 100 µl with 5 µl of tracking dye (0.1% bromophenol blue) and 30 µl markers were carefully layered on the top of the tube gels and electrophoresed for 4h at a constant current of 5 mA/gel. After the run the gels were stained in ethidium bromide (20 ng/ml) for 15 min and observed on a UV transilluminator, at 260 nm.

The nature of the fluorescent bands was confirmed by post-staining digestion of the gel with 20 µg/ml RNAse (Ribonuclease A from Bovine pancreas, Sigma Ltd.) in water ('low' salt) or in 0.3 M sodium chloride ('high' salt) and DNAse (Deoxyribonuclease, from beef pancreas Sigma Ltd.) 50 µg/ml in 0.3 M magnesium chloride.

Relative molecular mass of dsRNA was estimated by a graphical method using the curvelinear relationship between $\log_{10}$ Mr and electrophoretic mobilities

of dsRNA (or dsDNA) in polyacrylamide gels (Bozarth and Harley, 1976). A curvelinear regression of electrophoretic mobilities on $\log_{10}$ Mr of standards was done and the curve was fitted with the aid of a computer programme.

2.14 Analysis of viral coat protein

Capsid protein of the viruses were analysed by SDS-PAGE (polyacrylamide gel electrophoresis in the the presence of SDS), a method used for separation and identification of polypeptide chains (Maizel, 1966 ; Shapiro ***et al.,*** 1966 and Vinuela ***et al.,*** 1967). Highly purified virus preparations were used to analyse the coat protein.

The virus solution was dissociated by SDS treatment and the number of polypeptide bands and their Mr estimated using the method described by Weber and Osborn (1969).

The polyacrylamide (7.5%) tube gels were prepared by mixing 5 ml acrylamide (30%) plus Bis-acrylamide (0.8%) mixture, 2 ml of 1 M sodium phosphate buffer pH 7 , 12.7 ml distilled water, 0.2 ml SDS (10%), 0.2 ml fresh ammonium persulfate and 0.01-0.02 ml TEMED.

The virus preparation in 0.01 M sodium phosphate buffer pH 7 containing 10 g/l SDS and 1% 2-mercaptoethanol was heated at 100°C for 3 min. before

loading. The protein standards used, with their Mr (Shapiro ***et al.,*** 1967; Dunker and Rueckert, 1969; Weber and Osborn, 1969 and Laemmli, 1970) were : bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (20,000),trypsinogen (24,000) trypsin inhibitor (20,100) and lactalbumin (14,2000).The virus samples and markers were loaded after adding a drop of glycerol and 5% of bromophenol blue, and electrophoresed for 4 - 5 h at a constant current of 7 mA / gel.

The gels were stained in Coomassie Brilliant Blue solution, prepared by dissolving 1.25 g in a mixture of 454 ml 50% methanol and 46 ml glacial acetic acid and removing insoluble materials by filtration. Staining was at room temperature for 2 - 3h. Gels were then thoroughly washed in distilled water and placed in destaining solution (50 ml methanol, 75 ml acetic acid and 875 ml distilled water) overnight.

The length of the gels before and after staining were measured, and also the positions of the blue protein zones were recorded. The mobility of the proteins was calculated using the method of Weber and Osborn (1969).

$$\text{Relative mobility} = \frac{\text{distance of protein migration}}{\text{length after staining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

The Mr of virus protein were obtained by estimation from the linear regression of electrophoretic mobility on $\log_{10}$ Mr of the standards as described for dsRNA.

2.15 Electron microscopy

Leaf squashes and partially or highly purified virus preparations were examined in a JOEL JEM 100 CX transmission electron microscope. The negative staining technique was used (Brenner and Horne, 1959)which involved mixing virus preparation with a solution of stain, *dodeca*-tungstophosphoric acid (PTA) at a concentration of 10-20g/1 (w/v) adjusted to the desired pH. Occasionally uranyl acetate (20g/1) was used, unadjusted for pH.

Leaf squashes were prepared by slicing and grinding a leaf piece from an infected plant on a clean glass slide in a drop of sterile water similar to the method of Walkey and Webb (1968). A small quantity of sap was collected by squeezing with a second glass slide onto the macerated tissue. A drop of this extract, (or a purified virus preparation) was placed on an electron microscope grid coated with collodion (1% nitrocellulose) or 0.8% formvar which in some cases was strengthened with carbon. Excess liquid was then removed with filter paper and the grid briefly allowed to air-dry. After

staining the grids were then examined for virus particles in the electron microscope.

2.16 *In vitro* cultures, heat therapy and chemotherapy experiments

Heat treatment applied in various ways has proved a successful method for the cure of many plant virus diseases (Nyland and Goheen, 1969). Heat treatment may be followed by tissue culture of the vegetative meristem (or shoot tips) to provide virus free plants (Hollings, 1965). Virus also has been eliminated from dormant vegetative tissues by subjecting them to high temperatures (Kunkel, 1935 ; Kassanis, 1950 ; Nyland, 1959). In addition, virus has also been successfully eradicated from germinating seeds and isolated embryos grown in tissue culture, subjected to heat treatment ***in vitro*** (Cooper and Walkey, 1978).

Seeds of ***C. quinoa, Nicotiana rustica*** and ***Pastinaca sativa*** cv. White Gem were used to initiate cultures in the present studies. The medium used was essentially the same as that used by Cooper and Walkey (1978) to raise ***N. rustica*** seed cultures but a solid agar medium was used instead of a liquid medium. Infected seeds of ***C. quinoa*** and ***N. rustica*** were surface sterilized in 5% sodium hypochlorite containing 1% Tween 20 for 15 min Seeds were then thoroughly rinsed in three changes of

sterile distilled water before being introduced to the culture medium.

Parsnip seeds were sterilized in 5% sodium hypochlorite (containig 1% Tween 20) for 5 - 10 min, and washed in three changes of sterile distilled water. Seed were then dehusked using a sterile scalpel and a pair of forceps under a lamina flow hood. Dehusked seeds were then introduced aseptically to the medium after a quick rinse in 1% hypochlorite solution.

Media preparation

Half a sachet of mineral salt based on Murashige and Skoog's medium (commercially pre weighed and mixed to prepare 1 litre, Flow laboratories) and 30 g of sucrose were dissolved in 900 ml distilled water and the volume made up to 1litre. The pH of the medium was adjusted to 5.8 with N/NaOH and 6 g agar (tissue culture agar) was added and heated until the agar was dissolved. The medium was autoclaved for 15 min at 10^5Newtons/ m^2 pressure.

Disposable culture tubes of 75 x 25 mm or 15 cm glass tubes were used to maintain cultures and the cultures transferred to fresh medium at three week intervals when necessary.

2.17 General maintenance of glasshouses and growth cabinets

The following procedures were taken to prevent contamination with any unwanted viruses :

(i) disease free plants that were to be inoculated were raised on a separate bench, screened with insect proof nets.

(ii) weeds and plant debris were regularly removed and destroyed, and the benches were sprayed with Decon 75 (Decon Labs Ltd.) once a month.

(iii) Before infected plants flowered, they were moved below the glasshouse benching, in order to prevent the contamination of adjacent plants by infected pollen. Plants maintained in growth cabinets were covered with muslin to prevent cross contamination with pollen.

(iv) Infected plants were carefully handled so as to prevent accidental mechanical transmission. Hands and tools were washed thoroughly with soap immediately after handling the infected plants.

(v) Razor blades, scalpels, knives and scissors were soaked in solutions of decon 75 detergent and cleaned between use.

(vi) All non-disposable pots and trays were soaked in 5% (w/v)formaldehyde solution for at least one week and then thoroughly washed in detergent, and rinsed in water between use.

Pest and disease control

Glasshouse white fly (*Trialeurodes vaporar iorum*) red spider mites (*Tetranychus urticae*) and aphids (more than one species) were found to be common glasshouse pests and were controlled using the following pesticides :

Red spider mites	-	morestan (quinomethionate) dimethoate
Aphids	-	Temik 10-G Ambush C (Cypermethrin)
Whitefly	-	XL all insecticide (nicotine) and Ambush C

Moreover, glasshouses were regularly fumigated using nicotine shreds (Murphy Chemicals Ltd.) which controls a range of insect pests.

Fungal diseases occasionally found on plants in glasshouses and growth cabinets were controlled by spraying Benlate (systemic fungicide) or other fungicides. Slugs were controlled by using Slugits slug baits. (Murphy chemical Ltd.)

2.18 Cleaning of glassware and apparatus

All virus-contaminated glassware was thoroughly washed in hot water and autoclaved for 15 min at 10^5Newtons/m^2 pressure or, alternatively was soaked overnight in Decon 75. All blenders and centrifuge tubes used in purification were soaked in Decon 75 for at least two days before thorough washing in distilled water.

2.19 Chemicals

Unless otherwise stated all chemicals and reagents used were either from Sigma Company Ltd. or from BDH (British Drug House, England) and were of Analar grade.

EXPERIMENTS AND RESULTS

CHAPTER 3 Isolation and biological characterisation of SLRV.

3.1a Isolation and identification of SLRV from field grown parsnips

Attempts were made to isolate SLRV from field grown parsnip cv. White Gem plants with virus-like symptoms. Newly expanded young parsnip leaves were ground in inoculation buffer and indexed to at least two young *C. quinoa* test plants (Chapter 2.3). The presence of SLRV was suggested by the host reaction on these test plants and, subsequently, confirmed by agar gel double-diffusion serology (Chapter 2.11b) using an antiserum to SLRV type strain kindly supplied by Dr.A.F.Murant (Scottish Crops Research Institute, Invergowrie, Dundee). A series of antiserum and antigen dilutions were tested.

Out of 17 parsnip plants tested, seven (41%) were found to be infected with SLRV when indexed on *C. quinoa*. The isolates/plants were named as A,B,C,to Q and the results are summarized in **Table 1.**

The isolates D,F,G,I,K,L and P produced various degrees of chlorotic mottle and apical necrosis on *C. quinoa* . Antiserum precipitin titres of 1 / 256 were readily obtained for these seven isolates using *C. quinoa* sap as antigen source and the precipitin lines of all fused with each other, suggesting that they were closely

related serologically. However, the isolates differed in their virulence on *C. quinoa*.

Table 1 Identification of virus isolates from parsnip.

Isolates	Reaction on *C.quinoa*	Reaction to SLRV antiserum
A	+	-
B	-	-
C	-	-
D	+	+
E	+	-
F	+	+
G	+	+
H	-	-
I	+	+
J	-	-
K	+	+
L	+	+
M	-	-
N	-	-
O	-	-
P	+	+
Q	-	-

Sap from these isolates was also tested with an antiserum to arabis mosaic virus (AMV) which is

also transmitted by the nematode, ***X. diversicaudatum*** and is sometimes found in association with SLRV (Lister, 1964); no mixed SLRV/AMV infections, however, were found.

As also reported by Cooper (1981) no reaction was obtained against SLRV antiserum when crude parsnip sap was used.

Although the isolate A produced symptoms similar to those produced by SLRV in ***C. quinoa*** it failed to react with antisera to SLRV or AMV. This isolate was lost before an identification could be made. Isolate E produced about 10-15 local necrotic lesions of 0.5-1.0 mm in diameter on ***C. quinoa*** followed by systemic chlorotic mottle and flecks, two weeks after inoculation. This isolate was identified as parsnip yellow fleck virus (PYFV) by double-diffusion test using an antiserum to PYFV kindly supplied by Dr. D. G. A. Walkey (Institute of Horticulture Research, Wellesbourne).

The other parsnip plants ie; B, C, J, H, M N. O and Q were apparently virus-free although they may have been infected with viruses that were not sap-transmissible. A summary of the symptoms produced by the parsnip isolates on ***C. quinoa*** and the symptoms observed on their respective natural hosts is given in **Table 2.**

Table 2 Symptoms associated with SLRV in parsnip and *C. quinoa* .

Isolates/ plants	Symptoms in parsnips	Symptoms in *C. quinoa*
A	general chlorosis in young and old leaves	mild chlorotic mottle in systemic leaves
B	vein mosaic in older leaves and chlorotic spots in young leaves	no infection
C	slight mottling and crinkling	no infection
D	chlorotic spots in young leaves,older leaves distorted, necrosis along veins plant greatly stunted	severe systemic necrosis around growing points
E	plant severely stunted,necrosis along veins, leaf size greatly reduced	local necrotic spots systemic fleck and mottle
F	crinkling and chlorotic mottle in leaves	severe chlorotic mottle followed by apical necrosis
G	general yellowing of plant	systemic chlorotic mottle
H	necrotic spots along veins, plant stunted	no infection
I	crinkling and mottling, plant greatly stunted	systemic mottle,local chlorotic spots,severe necrosis around growing points
J	slight chlorosis on leaves	no infection
K	chlorotic flecks and slight mottling	systemic mottle and necrosis
L	crinkled leaves and stunting	systemic chlorotic mottle followed by necrosis

Contd...

M	mottling in leaves, and plant stunted	no infection
N	necrotic spots in older leaves	no infection
O	leaf mottle and plant stunted	no infection
P	severe chlorotic mottle in all leaves	systemic leaf mottle, necrosis around growing points
Q	leaf mottle and crinkling	no infection

3.1b Isolation and identification of SLRV from commercial seed packets of parsnips

Plants grown in the glasshouse from commercial seed packets of parsnip cvs. White Gem, Improved Hollow Crown and Tender and True were indexed on to *C. quinoa* using leaf inocula from young plants (Chapter 2.3). About 30 - 40 plants were tested individually, each on two *C. quinoa* plants. The results are shown in **Table 3.**

Parsnip seedlings grown under glasshouse conditions showed various degrees of leaf mottling and leaf spotting, and about 20 - 30% were found to be infected with SLRV. However, as in field plants, symptom expression was not always associated with SLRV or any other sap transmissible virus.

Table 3 The presence of SLRV in different parsnip cvs. from commercial seed packets

Parsnip cv.	Reaction in *C. quinoa*	Percentage infection
'White Gem'	+	24%
'Tender and True'	+	33.3%
'Improved Hollow Crown'	-	-

For futher studies three SLRV isolates and one PYFV (isolate E) were selected. The SLRV isolates were selected on the basis of their virulence in *C. quinoa* (**Fig.1**). These were isolates D and I from field plants and another isolate from a commercial packet of cv. White Gem seed which was designated as SLRV-R.

3.2 Host range and symptomatology of SLRV isolates

About 30 species in seven families were manually inoculated with infective extracts of *C. quinoa* leaves. Those species that did not show any symptoms in four weeks were back tested for symptomless infection, in inoculated and uninoculated leaves, on *C. quinoa.*

The range of symptoms, where present, consisted mainly of various degrees of chlorotic mottle and necrosis, local chlorotic or necrotic lesions leaf distortion, abnormal pigmentation (reddening of leaves) and stunting. The results are summarized in **Table 4.**

Table 4 Host range of parsnip isolates of SLRV

Plant species	SLRV isolates D	I	R
Chenopodiaceae			
C.quinoa	(CL),SM,SN	(CL),SM,SN	CL,SC,SN
C.amaranticolor L. Coste and Reyn	L,SM	L,SM	L,SM
C.murale L.	NL,SN,D	NL,SN,D	NL,SN,D
C.foetidium L.	L,SO	L,SO	L,SO
C.album L.	L,SO	L,SO	L,SO
Beta vulgaris 'Globe'	NL,S,R	NL,S,R	NL,S,R
Spinacea oleracea	(SM),L,SO	(SM),L,SO	(SM),L,SO
Umbelliferae			
Daucus carota	(CL) SO	-	-
Angelica sylvestris	-	-	-
Apeum graveolens	-	-	SO
Anethum graveolens	SO	-	SO
Coriandrum sativum	SO	(SN),SO	-
Solanaceae			
Nicotiana tabacum			
'White Burley'	L,SO	L,SO	L,SO
'Xanthi'	L,SO	L,SO	L,SO
N. clevelandii	L,SO(SM)	L,SO(SM)	L,SO(SM)
N. rustica	L,SO	L,SO	L,SO
N. debneyi	L,SO	L,SO	L,SO
N. sylvestris	L,SO	L,SO	L,SO
Datura stramonium	-	-	-
Petuna multiflora	L,SM	L,SM	L,SM
Cucurbitaceae			
Cucumis sativus			
'Parisienne Pickling'	L,SO(SM)	L,SO,(SM)	CL,SM
'Butchers Disease Resister'	-	-	-
'Ridge Crystal Apple'	-	-	-
'Marketer'	L,SO(SM)	L,SO(SM)	CL,SM
Amaranthaceae			
Gomphrena globosa	SO	SO	SO
Leguminoceae			
Phaseolus vulgaris 'Prince'	-	-	-
P. multiflourus	-	-	-
Vicia faba	-	-	-
Pisum sativum ''Meteor'	-	-	-
Cruciferaceae			
Brassica campestris subsp. 'Chinensis'	-	-	-
Lactuca sativa	-	-	-

CL, Chlorotic local lesion
SC, systemic chlorosis
SO, Symptomless systemic infection
SM, Systemic mottle
SN, Systemic necrosis
NL, Necrotic local lesion
L, Local infection

(), occasional symptom
C, chlorosis
R, reddening of leaves
D, distortion of leaves
-, no infection
N, necrosis

Fig. 1 *C. quinoa* infected with SLRV isolates. From left to right; SLRV-I, SLRV-D, SLRV-R and healthy

Fig. 2 Chlorotic local lesions induced by SLRV-D in carrot.

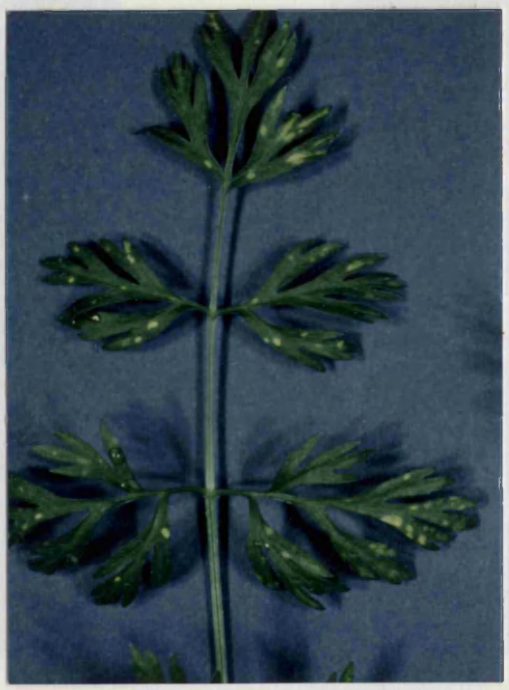


Fig. 3a Beetroot plants infected with SLRV-R (left) showing reduction in leaf size, distortion and reddening. Healthy plants on right.

Fig. 3b Close up of infected beetroot leaf showing necrotic ringspot symptoms. Healthy leaf on right

FIG. 4 *C. murale* with SLRV-D (right) showing apical necrosis and stunting when compared to healthy plants (left).Photograph was taken three weeks after inoculation.

Fig. 5 Systemic symptoms induced by SLRV-D in cucumber

The results showed that the three SLRV isclates from parsnips had wide host ranges which were typical for this virus (Lister, 1964). Differences in virulence and symptom expression of the three isolates were found in the following test hosts.

C.quinoa - This was the most sensitive herbaceous host tested for SLRV. The infection of SLRV isolates in this host clearly showed that they differed in their virulence. In a separate experiment, each isolate was inoculated to a uniform set of six, four week old *C. quinoa* plants. The inoculation buffer, carborundum and the amount of celite were kept the same for all three inocula. Another set of six plants of the same host were inoculated only with inoculation buffer containing celite and carborundum and all inoculated plants were then randomised in a glasshouse and maintained at 20 ± 2°C.

Six weeks after inoculation the heights of plants from compost level to growing point were recorded.

A second experiment was done in which the age of the inoculated plants was changed from 4 weeks (Expt.1) to six weeks (Expt.2). The results of both experiments are given in **Table 5.**

Table 5 Mean heights of *C.quinoa* infected with 3 SLRV isolates, Expt. 1, six week old plants and Expt. 2, 4 week old plants

Isolate	Expt. 1 Mean Height (cm)	Expt. 2 Mean Height (cm)
I	16.34(a)	26.93(a)
D	22.94(a)	35.37
R	26.26(b)	40.55
control	38.70	46.33

(a) significantly different from control($P<0.01$)
(b) significantly different from control($P<0.05$)

The results of experiment 1 showed that plants infected with all three SLRV isolates were significantly different in height from the healthy controls. But, no significant difference ($P > 0.05$) in height was found between isolates D & I, R & I or D & R.

The results also indicated that the effects of SLRV isolates on growth were more pronounced when the plants were younger at inoculation (experiment 1). Plants inoculated with SLRV-I, however, showed a severe necrosis around the growing point whatever age they were inoculated. The isolate D and R showed a moderate level of necrosis when inoculated to 4 week old plants but neither of these isolates induced necrosis when inoculated to 6 week old plants (**Fig. 1**). In general, all three isolates affected the growth significantly when compared

to controls. According to the heights of infected ***C.quinoa*** plants the virulence of the three isolates can be expressed as follows:

SLRV-R < SLRV-D < SLRV-I

(mild) (intermediate) (severe)

The 'mild' isolate SLRV-R regularly produced local chlorotic lesions on ***C. quinoa*** whereas the other two isolates occasionally did so, when conditions were optimum (young test plants, strong inoculum and a low post-inoculation temperature)

Cucumber - The isolate SLRV-R infected ***Cucumis sativus*** cvs. Parisienne Pickling and Marketer and readily produced chlorotic spots (about 1.0 - 1.5 mm in diameter) on inoculated cotyledons, about 20 per cotyledon being typical during routine inoculations. The local lesions were followed by a severe chlorotic mottle in second, third and fourth leaves although leaves produced later were symptomless.

In contrast, isolates I and D did not produce local lesions (although SLRV-D occasionally produced a few faint chlorotic spots). In winter a systemic chlorotic mottle developed in second and third leaves, although at other times of the year infected plants were normally symptomless.

The length of time taken to show symptoms in cucumber was different for different isolates. SLRV-R took six days to show local infection on cotyledons and about two weeks to produce systemic mottle. The other two isolates took about three weeks to show systemic symptoms, if present.

These observations indicated that the virulence of SLRV isolates varied with the host species. Although SLRV-R was mild in *C. quinoa* **(Table 5)** it produced severe symptoms in cucumber, suggesting that symptom expression depended on the genotype of both host and virus isolate.

The host ranges of the three isolates also differed **(Table 4)** especially in their ability to infect umbelliferous test plants. Among the seven umbelliferous species tested, SLRV-I infected coriander only, whereas SLRV-D infected coriander and dill readily but carrot with difficulty. Only two out of five carrot plants became infected with SLRV-D. On the other hand, several attempts to infect coriander with SLRV-R have failed and celery was infected with difficulty (only two out of five plants); dill, however, was readily infected.

In general, it seemed that umbelliferous species were quite resistant to SLRV isolates when mechanically inoculated with infected *C.quinoa* sap although natural infections have been recorded in many

umbelliferous crops including parsley (Henson and Campbell,1979) and celery (Walkey and Mitchell, 1969).

3.3 Effect of different pre-inoculation temperatures on the susceptibility of different hosts to infection by SLRV-D and SLRV-R

A wide range of environmental factors including light and temperature, are known to influence the pre or post-inoculation susceptibility of plants to infection (Matthews, 1981). Manipulation of the environment to increase infection of test plants may sometimes result in improved yields of virus during purification (Hicks,1979). Accordingly, a series of experiments were done to see if changes in temperature before and after inoculation, and light before inoculation, influenced susceptibility of *C. quinoa* or cucumber to SLRV.

Effect of short periods of pre-inoculation temperature

C. quinoa which was found to be the most susceptible host was used in these experiments. No satisfactory local lesion host for SLRV isolates was identified **(Table 4)** although Murant (1974) recomended *C. murale* . In the case of SLRV-R, *C. sativus* cv. Parisienne Pickling or Marketer could be used as a local lesion host as it produced distinct countable chlorotic local lesions although the virus did become systemic later.

Uniform six week old *C. quinoa* plants were kept at two different temperatures, 18 ± 1°C and 26 ± 1°C in growth cabinets (Fisons) illuminated with a light intensity of 7,600 lux for 16 h/day. This treatment was given for 48h , and plants were inoculated with leaf extracts of *C. quinoa* infected with SLRV-D or R. All plants were kept in a glasshouse at 20 ± 2°C after inoculation and monitored for symptom production. The results are shown in **Table 6.**

It was found that the infection by SLRV had decreased 30 - 40% in the plants given a 48h high temperature (26°C) treatment, suggesting that the plants had a reduced susceptibility when exposed to this temperature shortly before inoculation. The results were significant ($\chi^2 = 4.9$, $P \leq 0.03$ for SLRV-D and $\chi^2 = 6.23$, $0.01 < P < 0.025$ for SLRV-R)

Table 6 Effect of pre-inoculation temperature on the susceptibility of *C.quinoa* to infection by SLRV-D and SLRV-R

	Infectivity(a)	
Treatment	SLRV-D	SLRV-R
18±1°C / 20±2°C	20/20	20/20
26±1°C / 20±2°C	14/20	13/20

(a) No. infected / No. inoculated

The same experiment was repeated using SLRV-R/cucumber cv. Parisienne Pickling combination. Ten days old cucumber seedlings with two cotyledons stage were inoculated with buffered leaf sap of *C. quinoa* infected with SLRV-R. The infection was assessed by counting the total number of local lesions on both cotyledons in ten cucumber seedlings and the results are given in **Table 7**.

Table 7 Effect of pre-inoculation temperature on the susceptibility of *C. sativus* cv Parisienne Pickling to SLRV-R

	* Infectivity	
	18±1°C/ 20±2°C	26±1°C/20±2°C
	12	12
	11	08
	09	10
	17	07
	15	08
	36	07
	27	05
	28	07
	15	09
	14	07
Mean/Plant	18.4	8.0

* Lesions per plant

This experiment was repeated two more times and the results were found to be significant ($P < 0.05$) in each case, with more lesions produced on plants grown at 18°C before inoculation.

It is evident from these results that the effects of the temperature treatments on infection was similar for both host species tested. The variations in lesion numbers on plants grown at 26°C were probably due

to an effect on the host rather than the virus.

3.4 Effect of pre-inoculation dark period on the susceptibility of *C. quinoa* and cucumber to infection by SLRV-R

Twenty uniform plants of six week old *C. quinoa* were kept in a dark box in the glasshouse at 20 ± 2°C for 24h prior to inoculation. A control set of 20 plants of the same species was kept just outside the dark box in the same glasshouse with a 16h photoperiod. Both sets of plants were inoculated 24h after the treatment with extracts of *C. quinoa* leaves infected with SLRV-R, and the plants were randomised in the same glasshouse and kept for two weeks for symptom development. The results are shown in **Table 8**.

Table 8 Effect of pre-inoculation dark treatment on the the susceptibility of *C. quinoa* to SLRV-R

Treatment	* Infectivity
24h dark at 20°C	20/20 (in 9 days)
controls at 20°C	12/20 (in 9 days)
with no dark treatment	15/20 (in 14days)

* No.infected / No.inoculated

The results are significant ($\chi^2 = 3.6$, $0.05<P<0.1$) and it is clear that there was a reduction in the susceptibility of plants that were not given a pre-inoculation dark treatment,and also a delay in symptom

expression. However, when this treatment was repeated with 3 - 4 week old *C. quinoa* seedlings 17 out of 20 plants became infected (not significant, $\chi^2 = 1.4$, $P > 0.3$) without a pre-dark treatment. Therefore, it seems the dark treatment is more important for the susceptibility of older plants to infection by SLRV. Probably the older plants are 'harder' and the dark treatment may be more effective in increasing susceptibility in such plants (Bawden and Roberts, 1948).

Further investigations were made to study the effect of pre-dark treatment on the susceptibility of cucumber to infection by SLRV-R. A 24h pre-inoculation dark treatment produced slightly more lesions than those not given a dark treatment, but the difference was not significant ($P>0.01$).

However,when the experiment was repeated increasing the length of the pre-inoculation dark period to $2_{1/2}$ days, the number of lesions in cucumber was markedly reduced in the treated plants when compared to the controls. The mean no. of lesions/plant in treated and controls were 5.2 and 11.0, respectively ($P < 0.05$).

These results indicated, therefore that a 24h pre-inoculation dark treatment had no effect on the susceptibility of cucumber to SLRV but a longer period of the same treatment reduced susceptibility indicating that the effects depend on the host/virus combination.

3.5 Effect of pre-inoculation chilling period on the susceptibility of cucumber to infection by SLRV-R

One tray containing 12 seedlings of *C. sativus* cv. Parisienne Pickling grown in a glasshouse at 20 - 22°C, was kept in a cold room at 4°C and another tray (control) was kept at room temperature for 4h prior to inoculation (light factor was neglected). The seedlings were inoculated as in previous experiments (Section 3.3). Both trays were transferred to a growth cabinet at 18°C with a 16h photoperiod and a light intensity of 7,600 lux. Numbers of lesions were counted eight days after inoculation and the results are shown in **Table 10.**

Table 10 Effect of pre-inoculation chilling on the susceptibility of cucumber to infection by SLRV-R

Treatment	Total No.of lesions in 12 plants	Mean/plant
4°C / 18±1°C 4h 8 days	77	6
20°C / 18±1°C 4h 8 days	59	5

The results were not significant ($P > 0.05$) although, the total number of lesions were 30% higher when the plants were pre-chilled prior to inoculation.

3.6 Effect of growing temperature on the susceptibility of cucumber to infection by SLRV-R

In this experiment, cucumber seeds were germinated and grown in growth cabinets at two

temperatures (18 ± 1°C and 25 ± 1°C). Growth cabinets were illuminated (7,600 lux) for 16h/day and one tray sown with *C. sativus* cv. Marketer (as cv. Parisienne Pickling was unavailable due to a crop failure, all subsequent experiments were done using the cv. Marketer), was kept at 18°C. The second tray to be kept at 26°C, was sown four days after the first tray, in order to balance the age of both sets of plants (fully expanded cotyledons) at inoculation.

When seedlings were at their two cotyledon stage they were inoculated with sap extracts of systemically-infected *C. quinoa* leaves. All plants were kept at 18°C after inoculation. Total number of lesions in 12 seedlings were counted ten days after inoculation and the results are given in **Table 11.**

Table 11 Effect of growing temperature on the susceptibility of cucumber to infection by SLRV-R

Growing temperature	Total No. of lesions in 12 plants	Mean/plant
26°C/18°C	82	7
18°C/18°C	107	9

The results indicated that there was no significant difference ($P > 0.05$) between lesion numbers in plants grown at 26°C and 18°C although the total

numbers of lesions produced were 30% higher at 18°C.

However,when cucumbers were grown at 32°C a total of eight lesions in 12 seedlings were produced compared to 98 ($P<0.01$) lesions on 12 plants grown at 18°C. Thus, growing plants at high temperatures before inoculation decreased susceptibility of cucumber to SLRV.

Furthermore, in an earlier experiment cucumbers given 48h at either 26°C or 18°C before inoculation (after continuous growth at 20-22°C), the plants exposed to higher temperature had decreased susceptibility (**Table 7**).

3.7 Effect of different short periods post-inoculation temperatures on infection of *C. quinoa* by SLRV-R.

In all previous experiments the environmental factors applied were prior to inoculation with SLRV. In the next few experiments the effects of temperature on SLRV infection after inoculation were investigated.

Uniform sets of 20, six-week old ***C.quinoa*** plants previously kept in dark for 24h were inoculated with buffered extracts of ***C. quinoa*** leaves (1:1 / w:v) infected with SLRV-R. After inoculation one set of plants was kept in a growth cabinet at 18°C (16h photoperiod) and the other was kept at 26°C with the same photoperiod,

illuminated with 7,600 lux. Inoculated plants were covered with a damp sheet of newspaper for 24h. The experiment was repeated using post-inoculation temperatures of 18°C and 32°C and also 18°C and 4°C. Only two temperatures were tested at one time and treatments were given for 24 h.

All trays were transferred to glasshouse conditions at 20°C immediately after treatment and kept for 2 - 3 weeks for symptom development. The results are shown in **Table 12.**

Table 12 Effect of short periods of different post-inoculation temperature on the infection of *C. quinoa* by SLRV.

Post-inoculation treatment	* Infection
24 h / 2 weeks	
1. 4°C / 20±2°C	20/20
18°C / 20±2°C	20/20
2. 26°C / 20±2°C	20/20
18°C / 20±2°C	20/20
3. 32°C / 20±2°C	6/20
18°C / 20±2°C	20/20

* No. infected / No. inoculated

The results indicated that although the exposure of plants to extremely low temperature (4°C) soon after inoculation did not noticably affect the infection, high temperatures (32 ± 1°C)for 24h immediately after inoculation significantly reduced ($\chi^2 = 18.6$ $P < 0.01$) infection of *C. quinoa*

The fact that six out of 20 plants became infected when the plants were kept at 32°C soon after inoculation, suggests that some virus particles were able to enter the host and establish infection in the plant. However, many particles may have been inactivated by the high temperatures before they were able to enter the host and establish infection.

In the next experiment, the inoculated plants were kept at 18°C for 48h immediately after inoculation allowing sufficient time for the virus to enter the host. Half of the plants were then returned to the growth cabinet at 32°C with 12 h photoperiod and about 7,000 lux illumination.

No symptoms could be detected after two weeks at 32°C and, therefore, the plants were back tested on *C. quinoa* seedlings for symptomless infection. The results are shown in **Table 13.**

Table 13 Effect of different post-inoculation temperatures on the infection of *C. quinoa* by SLRV-R

Treatment	*Infection
18°C / 18°C / 18°C	20/20
18°C / 18°C / 32°C dark / 48h / 2 weeks 24 h	0/20

* No. infected / No. inoculated

It was evident that although the immediate post-inoculation temperature (18°C) was favourable for virus to enter the host, the temperature at which the host plants were subsequently kept appeared to have inhibited some later stage in virus multiplication, as virus could not be detected in plants, after two weeks at 32°C.

After two weeks at 32°C the plants were again transferred to 18°C and kept for another 3 - 4 weeks, but they showed neither symptoms nor were infected symptomlessly. However, four out of ten plants whose roots were indexed to *C. quinoa* seedlings still contained SLRV. It is possible that the roots of these plants were at a sufficiently lower temperature to support some virus replication. Furthermore, the failure to detect virus in foliage of these plants suggested that movement of virus from roots to leaves was affected.

3.8 Effect of long term post-inoculation temperatures on the infection of SLRV-R in cucumber and *C. quinoa*

In the next experiment, *C. quinoa* plants inoculated with SLRV-R were kept continuously at 26°C soon after inoculation and compared with plants kept at 18°C. It was found, however, that all 20 plants inoculated became infected and showed symptoms at both temperatures.

In contrast, when cucumber cv. Marketer seedlings were grown at 20°C inoculated and kept at 26°C for ten days immediately after inoculation, no local lesions or systemic symptoms could be detected, although about 58% of inoculated plants were infected. (**Table 14**)

Table 14 Effect of post-inoculation temperature on the infection of SLRV-R in cucumber

Treatment	Infection
20°C / 18°C 24h dark / 10 days	130(a)
20°C / 26°C	7/12(b)

(a) total no. of lesions in 12 plants
(b) symptomless infection, no.infected/no.inoculated

The results of these experiment showed that the effect of post-inoculation temperature on SLRV infection varied with the host species. Infection of ***C.quinoa*** by SLRV seemed to be less sensitive to the higher temperatures than cucumber, as all 20 plants became infected and developed symptoms, whereas no local lesions or systemic symptoms could be found in cucumber at this temperature (26°C). The results also indicated that ***C. quinoa*** was probably more susceptible to SLRV than cucumber, and this host was chosen, therefore, to propagate SLRV .

Chapter 4 Physical, chemical and serological properties of SLRV isolates from parsnips

4.1 *In vitro* properties of SLRV-D, SLRV-I and SLRV-R.

Dilution end point (DEP), TIP and LIV of the three isolates were determined using ***C.quinoa*** as source plant.Inocula were made at the rate of 1:0.5 / w:v in inoculation buffer and tested on ***C.quinoa*** as described in Chapter 2.9. **Table 15** shows a comparision of the physical properties of SLRV isolates.

Table 15 ***In vitro*** properties of SLRV isolates from parsnip.

Isolate	TIP °C	DEP	LIV(days)
SLRV-I			
Expt. 1	55-60	10^{-2}- 10^{-3}	8-12 at room temperature
Expt. 2	57-59	1/125-1/625	100+ at 4°C
SLRV-D			
Expt. 1	55-60	10^{-2}- 10^{-3}	8-12 at room temperature
Expt. 2	57-59	1/125-1/625	not tested
SLRV-R			
Expt.1	50-55	10^{-3}- 10^{-4}	4-8 days
Expt.2	54-57	1/625-1/3125	not tested

The results showed that whereas, previously the host range and virulence of the isolates D and I in ***C. quinoa*** varied slightly, the physical properties did not show any difference. However, both isolates differed

slightly from isolate R with respect to physical properties. The isolate R apparently occurred in greater concentration in *C. quinoa* but was slightly more unstable at room temperature. The *in vitro* properties of these SLRV isolates were comparable with the published data (Lister, 1964; Cooper, 1981 ; Hicks *et al.*, 1986)

4.2 Purification of SLRV

Obtaining a 'pure' culture of SLRV isolates-R and D

Leaves of *C. murale* seedlings were inoculated with leaf extracts of *C. quinoa* infected with SLRV-R. When local lesions (necrotic spots) were formed in 7 - 9 days, individual well-separated lesions were used to establish single lesion isolate of SLRV-R as described in Chapter (2.4). After three successive single lesion subcultures in *C. murale* the virus was bulked in *C. quinoa* seedlings. The same method was used to obtain a pure culture of SLRV-D. The single lesion isolates were regarded as 'pure' cultures although variants may occur even in cultures established from single lesions.

4.2a Effect of two clarification methods on the yield of SLRV-D

Uniform six week old *C. quinoa* plants were inoculated with SLRV-D and grown in a glasshouse at 20-24°C. Systemically-infected leaves were harvested 10 days

after inoculation and the virus was purified from 50g of materials using two clarifying agents, HCP and n-butanol (Chapter 2.10). After the first cycle of differential centrifugation, the partially-purified preparation was assayed for infectivity end point in *C. quinoa* seedlings and the antigen DEP determined in gel double-diffusion tests using a ten fold series of dilutions of the virus preparation, and an antiserum to SLRV type isolate. The results are summarized in **Table 16.**

Table 16 Effect of two clarification methods on the purity and infectivity of SLRV-D

method of clarification	*260/280	infectivity end - point *C.quinoa*	DEP in in gel-diffusion (b)
HCP	1.0	10^{-2}-10^{-3}	10^{-2}
n-butanol	1.45	10^{-3}-10^{-4}	10^{-2}

* values uncorrected for light scattering
(b) The antiserum dilution was 1/8

The results indicated that a relatively high yield of virus could be obtained when n-butanol was used. The experiment was repeated with the same isolate many times and similar results obtained. Although there were no differences in DEP within the range of antigen dilutions tested, the infectivity end-point was higher for the preparation obtained after n-butanol clarification.

Moreover, the 260/280 ratio was between 1.4 - 1.8, for n-butanol method whereas it was 1.0 - 1.2 for HCP method (**Figs.** 6a and **6b**).

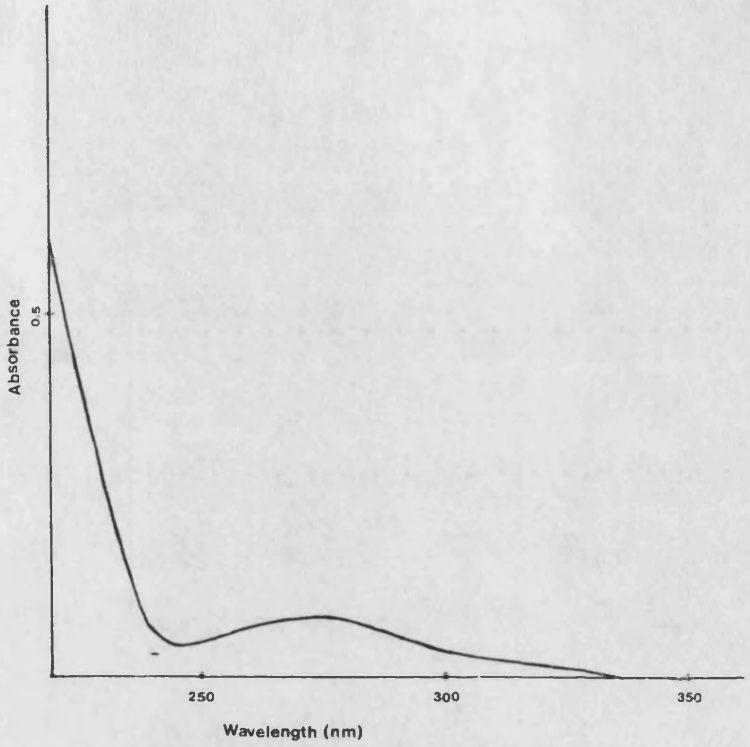
The A_{260}/A_{280} ratios for highly purified and fractionated particles of nepovirus were 0.7-0.8 (top component), 1.4-1.8 (middle component) and 1.7-1.8 (bottom component) according to Murant (1981). The above results suggested, therefore, that HCP preparations may contain more of the top component presumed to be mostly of empty shells (Murant, 1981). Observations of particles in the electron microscope confirmed the occurrence of more empty shells in HCP preparations when compared with butanol preparations (**Fig. 7a**).

Particles observed in the electron microscope were isometric with angular, often hexagonal outlines. Some particles were unpenetrated by the stain (2% PTA) whereas others were completely or partially penetrated. The diameter of SLRV particles was measured by comparison with lattice spacing for negatively stained catalase crystals (Wrigley, 1968).At least 100 particles were measured (at pH 6.5) and the diameter was found to be 26.9 ± 2.6 nm.

An effect of pH values of the stain was detected with much aggregation of particles at pH 8.0 (**Fig. 7b**) and 5.0. In contrast, at pH 6.5 (**Fig. 7c**) there was little evidence of aggregation.

Fig. 6 Absorption spectrum of partially-purified SLRV-D (diluted 1/100) (a) HCP clarification (b) butan-1-ol clarification

(a)



(b)

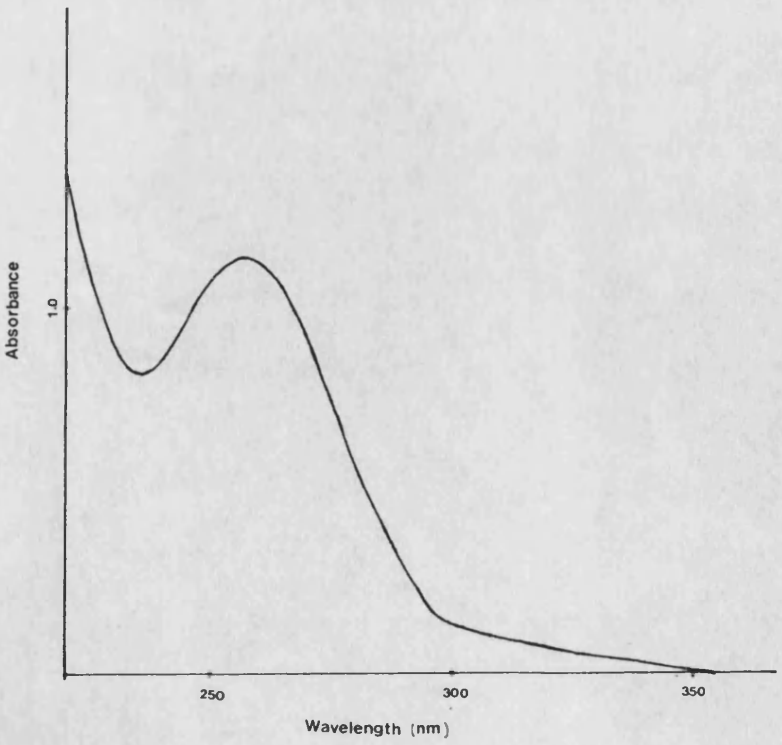
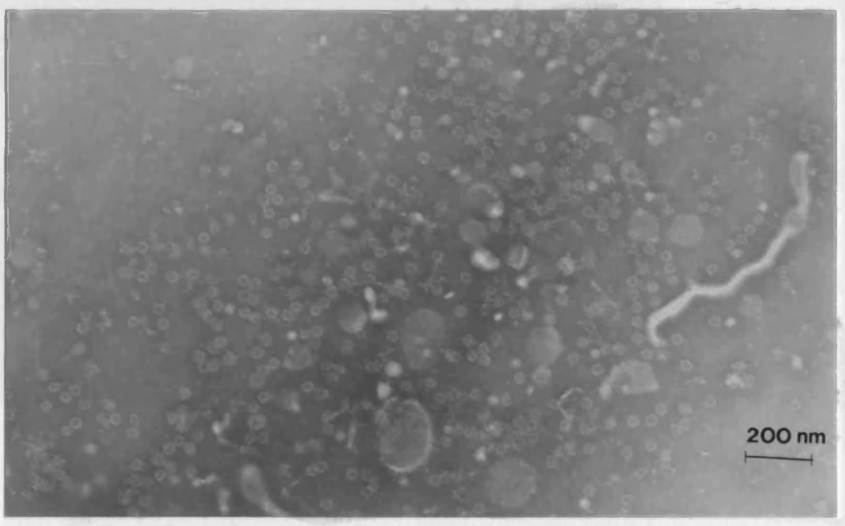
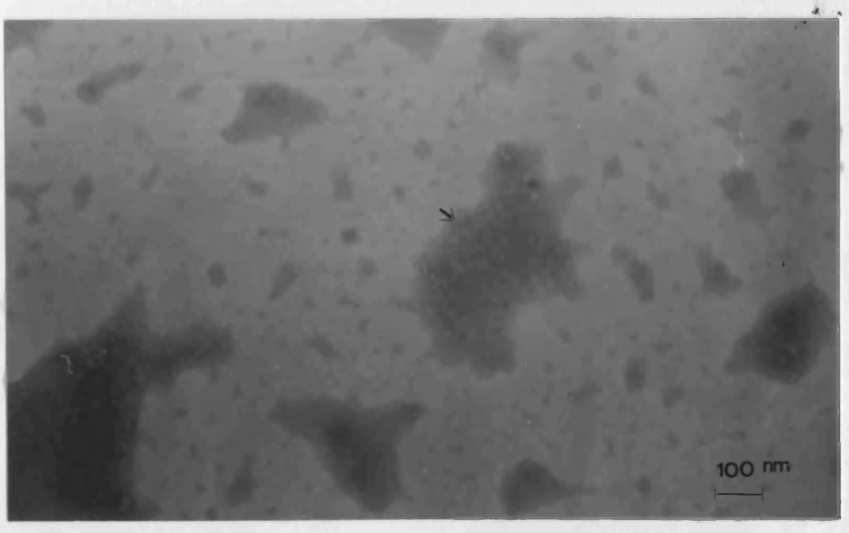


Fig. 7 Electron micrographs of partially-purified SLRV-D (clarified with HCP) in 2% *dodeca*--tungstophosphoric (a) 'empty' shells (pH 6.5) (b) aggregated particles (pH 8.0) (c) unaggregated particles (pH. 6.5)

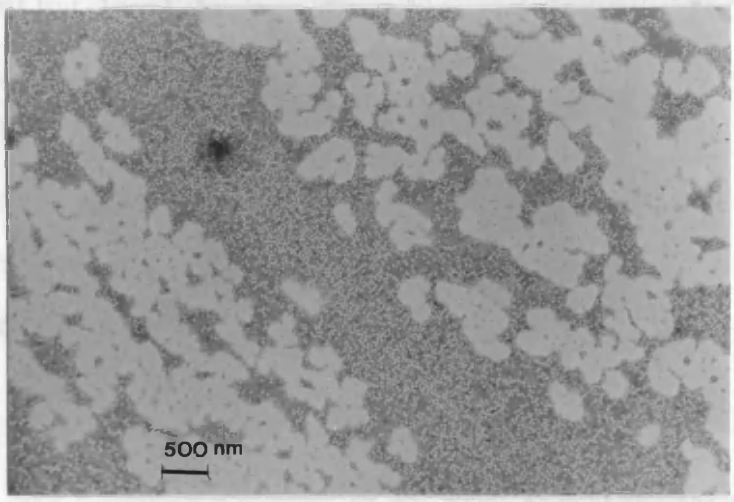
(a)



(b)



(c)



4.2b Effect of two clarification methods on the yield of SLRV-R

The same experiment (Section 4.2a) was repeated for SLRV-R but using cucumber to test infectivity by counting local lesions formed on cotyledons. In both methods, the virus was purified from 10g of cotyledons. After the PEG treatment (Chapter 2.10) pellets were resuspended overnight, in 1 ml of suspension buffer (0.01M phosphate buffer pH 7.8 for HCP method and 0.07M phosphate buffer pH 7 for butanol method) and centrifuged at low speed for 30 min.

The supernatants of both preparations were then made up to 2 ml using the same buffer and inoculated to 10 cucumber 'Marketer' seedlings. The plants were kept in growth cabinets at 18°C until lesions developed. The same preparations (undiluted) were used to test the DEP in gel diffusion and to obtain the absorption spectra. The results are given in **Table 17.**

Table 17 Effect of two purification methods on the yield of SLRV-R

Clarification method	260/280 (a)	Infectivity (b)	DEP in gel diffusion
HCP	0.92	29	10^{-2}-10^{-3}
n-butanol	1.4	43	10^{-2}-10^{-3}

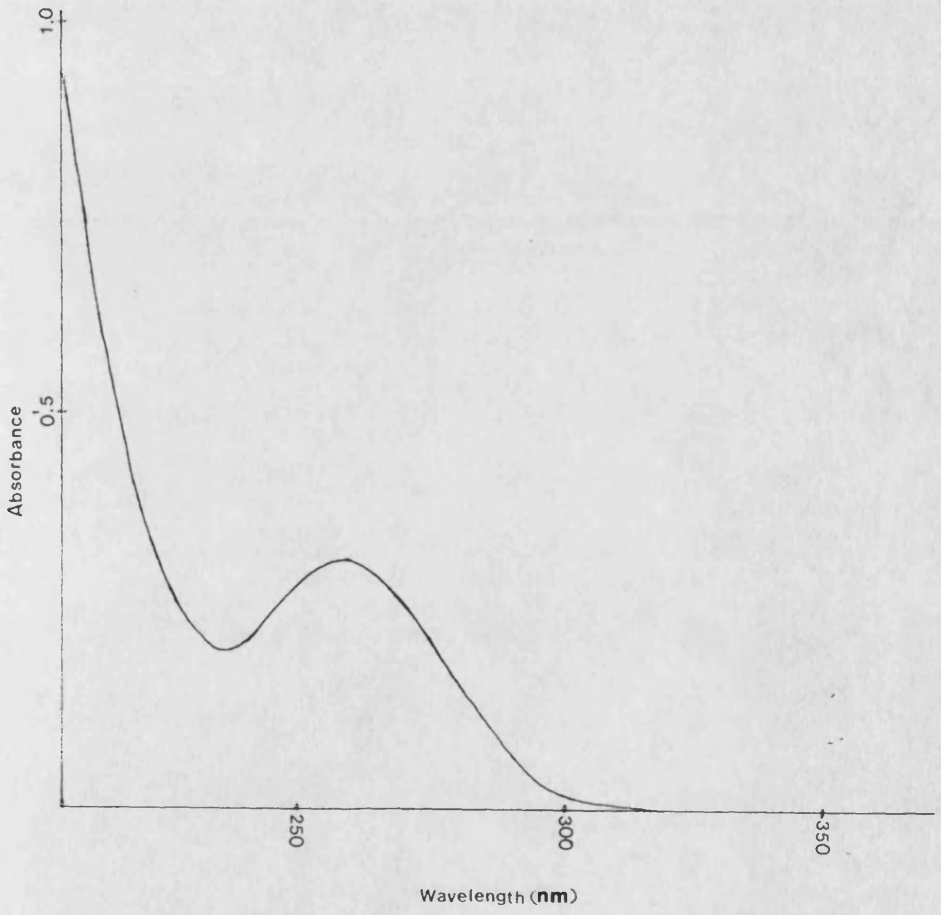
(a) values uncorrected for light scattering
(b) mean no. of lesions per plant

As with SLRV-D, higher yields of SLRV-R were obtained when the butanol method was used. This was supported by the UV absorption spectra and 260/280 ratio which was close to expected values (around 1.4 - 1.64, Murant, 1981). On the other hand, when HCP was used the maximum absorbance was about 270-275 nm. This resulted in a much lower 260/280 ratio in these preparations, possibly due, as with SLRV-D, to the presence of more empty shells; this could also explain the lower infectivity of HCP clarified preparations.

A characteristic feature often observed in the UV absorption spectra of butanol preparations was a very high peak which could be detected even when virus-free samples were analysed (**Fig.8**). The 260/280 ratio of 'healthy' preparations however, was equal to or higher than 2, thus indicating no virus particles in the preparation. For partially purified virus preparations the maximum absorbance was around 255-260 nm. The high absorbance at 260nm, however, made the calculation, of virus concentration unreliable.

Both HCP and butanol clarification produced, after column chromatography, preparations relatively free of host contaminants when examined in the electron microscope. However, the infectivity of butanol-clarified preparations was higher, and the 260 : 280 nm ratio closer to expected values, so this method was

Fig. 8 The uv absorption spectrum of a preparation from healthy *C. quinoa* , partially-purified by n-butanol (diluted to 1/100)



routinely used in subsequent purifications.

4.2c A comparison of the yield of SLRV-R purified from different hosts

Six-week old *C. quinoa* and 10 days old cucumber 'Marketer' seedlings were inoculated with buffered extracts of *C.quinoa* leaves infected with SLRV-R and grown at 18°C . *Chenopodium quinoa* leaves with clear symptoms were harvested 12 days after inoculation while with cucumber, inoculated cotyledons and systemically-infected leaves with mottling symptoms were harvested 10-16 days after inoculation. SLRV was purified from 10g of material by butanol clarification (Section 4.2) and the partially-purified virus preparation (before high speed centrifugation) was made up to 2 ml using the suspension buffer (Chapter 2.10) and assayed on 10 cucumber seedlings. The results are shown in **Table 18.**

Table 18 Infectivity and purity of SLRV purified from *C. quinoa* and cucumber (assayed on cucumber)

Host	Mean no. of lesion per plant	260/280 (a)
C.quinoa	32	1.4
cucumber	12	1.9

(a) data uncorrected for light scattering

The results showed that the infectivity of preparations from *C. quinoa* was higher than that of cucumber preparations. The high A_{280} value and 260/280 ratio obtained for the cucumber preparation could probably be due to contamination with some host materials.

These findings indicated that, *C. quinoa* was a better host than cucumber for purification of SLRV-R, although the latter was recommended as a propagation species for SLRV by Murant (1974).

4.2d The effect of harvesting time on the yield of SLRV-D in *C. quinoa*

Fifty three-week old *C.quinoa* plants, randomly arranged in a glasshouse at 24-30°C in summer. were inoculated with SLRV-D (in *C. quinoa* extracts). Infected leaves with symptoms were harvested at two different intervals 10 days and 17 days after inoculation. With each group the virus was purified from 40g of diseased material by n-butanol clarification. After the first differential centrifugation the partially-purified preparation was assayed for infectivity on *C. quinoa* and the DEP was also determined in gel double-diffusion tests using 1/8 dilution of an SLRV antiserum (type strain). The results are shown in **Table 19.**

Table 19 Effect of harvesting time on the yield of purified SLRV-D

Harvesting time(days)	Infectivity end-point in *C.quinoa*	DEP in gel diffusion	260/280
10	10^{-3}_ 10^{-4}	10^{-2}	1.83(a)
17	10^{-3}- 10^{-4}	10^{-2}	1.52(a)

(a) uncorrected for light scattering

The results showed that there was no significant difference in infectivity end-point after 10 or 17 day harvesting intervals and, therefore, harvesting times in this experiment had no obvious effect on the yield of SLRV-D in *C. quinoa.*

4.2e Further purification of SLRV-D and SLRV-R by controlled pore glass (CPG) chromatography.

After partial-purification of *C. quinoa* (100g) infected with SLRV-D or SLRV-R both isolates were subjected to further purification by CPG chromatography (Chapter 2.10). Comparisons were made between HCP and butanol clarified preparations.

Virus preparations from the HCP clarification commonly resulted in a single peak of absorbance (254nm, **Fig.9a**) which after concentration by high speed centrifugation, resulted in a clean preparation relatively free of host material when observed in the

electron microscope (EM). Moreover, there was no significant reduction in infectivity between preparations before and after column chromatography when equivalent dilutions were assayed on *C. quinoa*. Preparations occasionally produced two distinct peaks of which the first one contained apparently higher numbers of particles than that of peak 2 (**Table 20.**)

In contrast, butanol preparations typically produced two peaks (**Fig. 9b)** and both contained a high concentrations of particles (**Table 20**) with relatively high infectivity.

Table 20 The infectivity and number of virus particles associated with peaks of UV absorption after CPG chromatography.

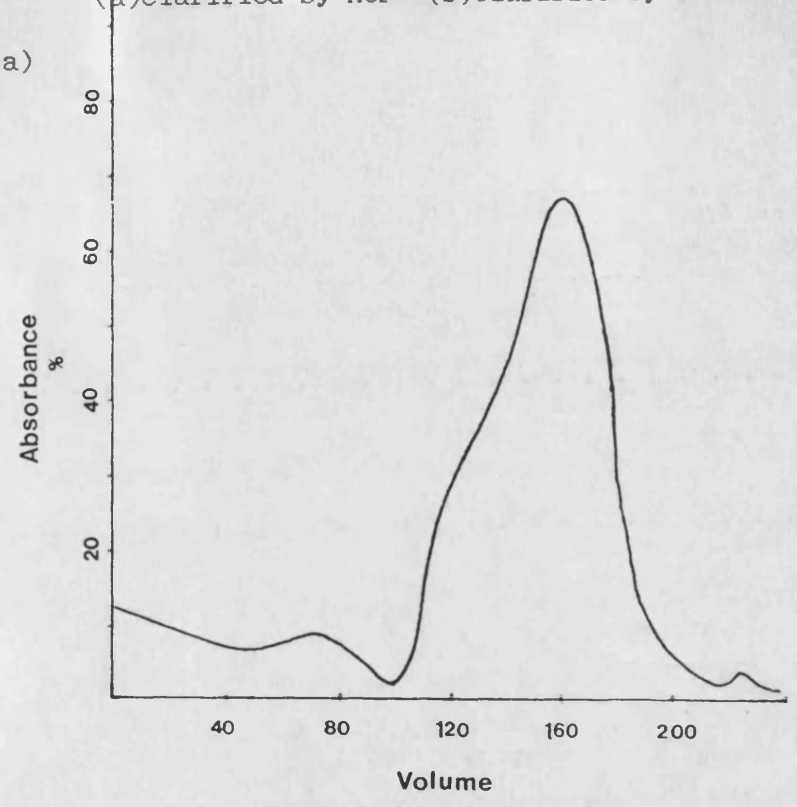
Peak	EM observation no.of particles	Infectivity
(i) HCP		
1	+ + +	+ + +
2 (rarely)	+ to + +	+ +
(ii) n-butanol		
1	+ + +	+ + +
2	+ + +	+ + +

+ + + high ++ moderate + low

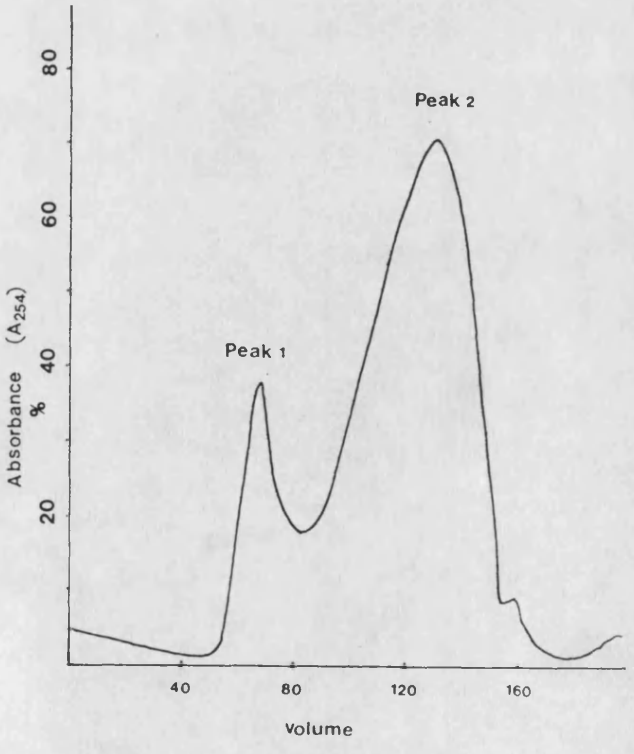
Butanol preparations occasionally produced three distinct peaks, and all three peaks contained SLRV, which when collected and centrifuged separately, showed no apparent differences in infectivity when assayed on

Fig. 9 Elution profile of SLRV-D clarified, and chromatographed on a CPG column (Void volume=90 ml) (a)clarified by HCP (b)clarified by butan-1-ol

(a)



(b)



electron microscope (EM). Moreover, there was no significant reduction in infectivity between preparations before and after column chromatography when equivalent dilutions were assayed on *C. quinoa*. Preparations occasionally produced two distinct peaks of which the first one contained apparently higher numbers of particles than that of peak 2 (**Table 20.**)

In contrast, butanol preparations typically produced two peaks (**Fig. 9b**) and both contained a high concentrations of particles (**Table 20**) with relatively high infectivity.

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2 (rarely)	+ to + +	+ +
(ii) n-butanol		
1	+ + +	+ + +
2	+ + +	+ + +

+ + + high ++ moderate + low

Butanol preparations occasionally produced three distinct peaks, and all three peaks contained SLRV, which when collected and centrifuged separately, showed no apparent differences in infectivity when assayed on

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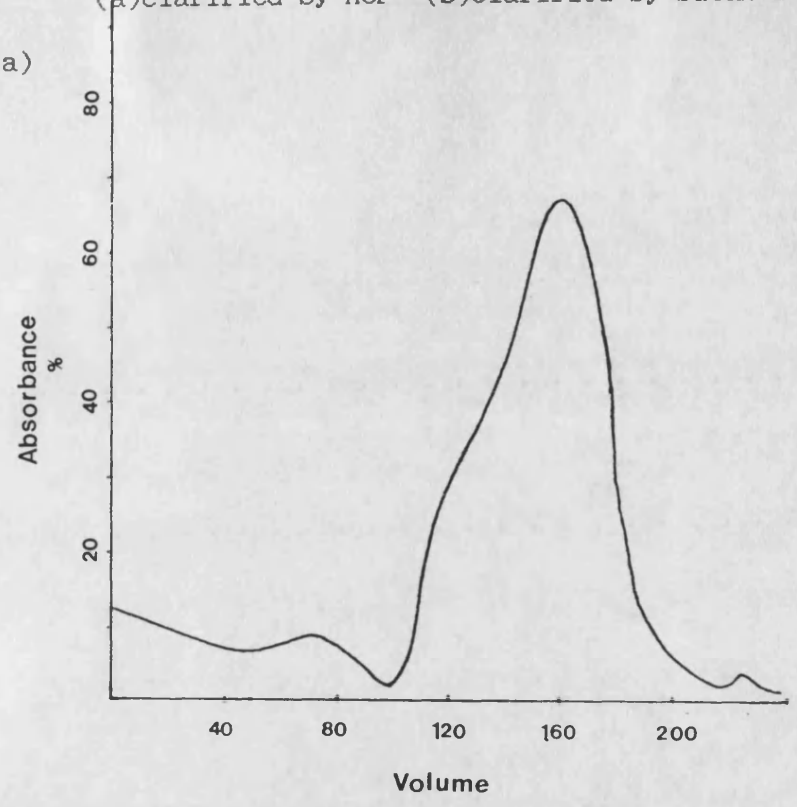
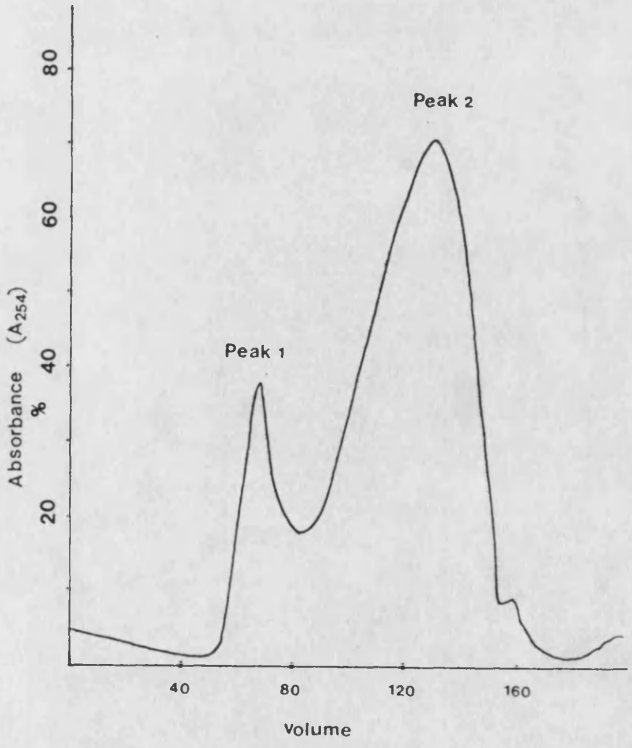
(a)



(b)



C.quinoa or in the number of particles seen in the electron microscope compared to samples before column chromatography.

With butanol clarified preparations it was observed in the electron microscope that passage through the CPG column sometimes failed to remove all impurities and preparations needed a second passage through the column. The UV absorption spectra of these highly purified preparations (after two cycles of column chromatography) however, did not show a very high absorbance at 260 nm (**Fig.10a**) compared with preparations before column or given one cycle of CPG chromatography. The infectivity or DEP in gel-diffusion was not apparently affected and this indicated that the high absorbance at 260 nm was due to a contaminating host component associated with butanol, removed only after two cycles of CPG chromatography.

4.3 Serology of SLRV-D, SLRV-R and C

For further characterisation of the parsnip isolates only SLRV-D and SLRV-R were selected. Antisera to these two isolates were produced using highly purified virus preparations (Chapter 2.10) in rabbits (Sandy half lop, Chapter 2.11a). In addition, an isolate of SLRV from the woody ornamental ***Caryopteris clandonsis*** designated as SLRV-C was used in comparative tests using an antiserum kindly provided by N.Ngamyeesoon (University of Bath).

Fig. 10a The uv absorption spectrum of SLRV-D (clarified by butan-1-ol) after two cycles of column chromatography and concentrated by high speed centrifugation (diluted to 1/100) a, peak 2 b,peak 1

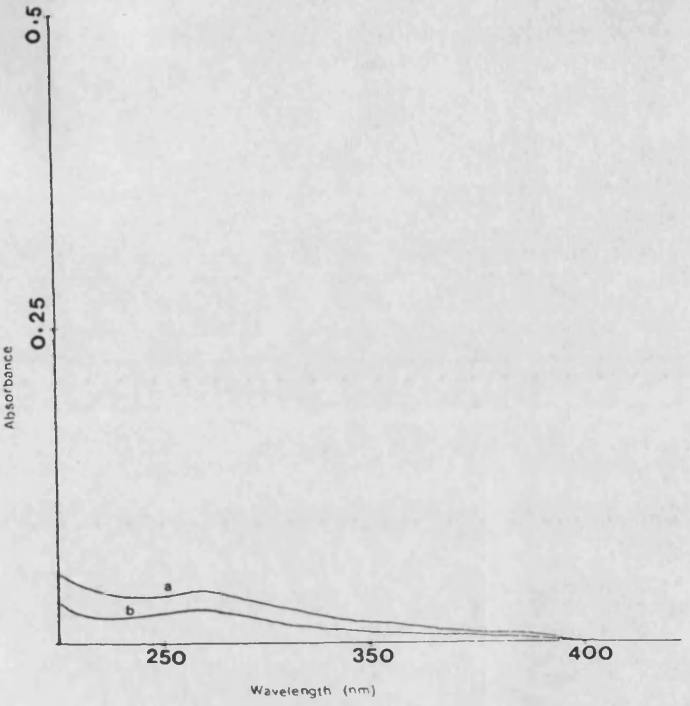


Fig. 10b Purified particles of SLRV-D by butanol method before column chromatography ('empty' shells arrowed)

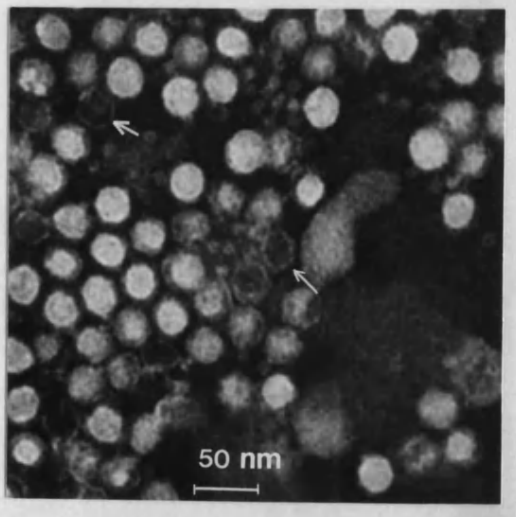
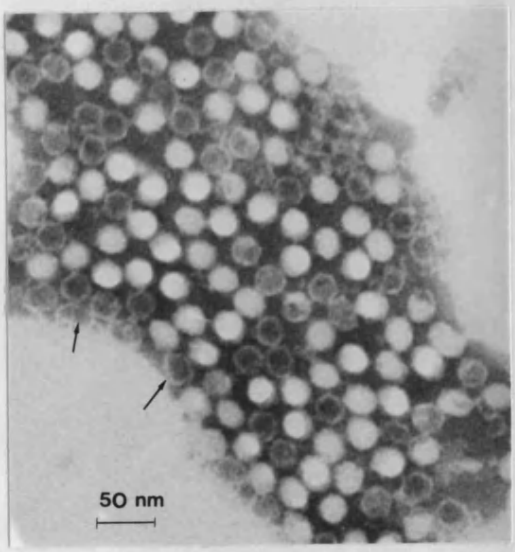


Fig. 10c SLRV-D particles after controlled pore glass chromatography



Titres of all three antisera in the gel double-diffusion tests were determined using a two-fold dilution series prepared in 0.85% saline (Chapter 2.11b). Antigens (undiluted crude sap) used were prepared from recently infected *C. quinoa* leaves (Chapter 2.11b) and tested undiluted, and at 1/4 dilution.

Antisera titres increased from the first to fifth bleed (ie from three to seven weeks after the first intravenous injection) and then decreased. The titre obtained for SLRV-D was 1/256 and that for SLRV-R was 1/128. The titre for SLRV-C was 1/32 with its homologous antiserum. Comparing a range of dilutions, it was found that the optimum ratio of crude virus antigen and the homologous antiserum to give sharp precipitin lines were 1:2/1:64 for SLRV-D and R and 1:2/1:16 for SLRV-C.

To determine serological cross reactivity between isolates, reciprocal gel-diffusion tests were done. The results are shown in **Table 21** and **Figs 11** and **12**.

Table 21 Results of reciprocal gel-diffusion test.

Virus	SLRV Antiserum R	D	C
SLRV-R	* 128	128	64
SLRV-D	128	256	64
SLRV-C	64	64	32

* reciprocal of antiserum titre (two-fold dilutions)

Fig 11 Reaction of SLRV isolates against antiserum to SLRV-D (centre well), (a) SLRV-D; (b) SLRV-C; (c) healthy.

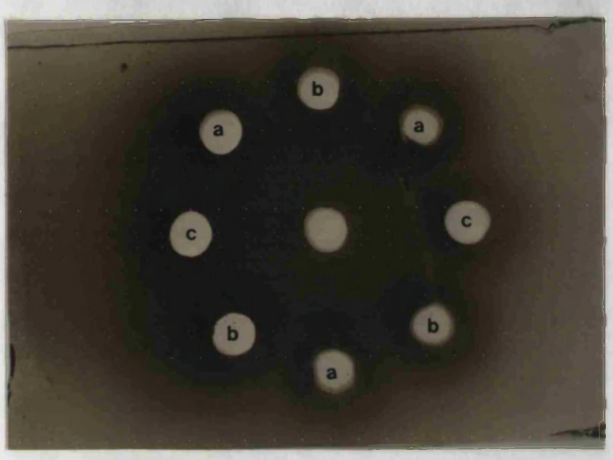


Fig. 12 Reaction of SLRV isolate against antiserum to SLRV-C (centre well) a,SLRV-D b, SLRV-C c, healthy

No obvious spurs were formed between antigens SLRV-R/SLRV-D, SLRV-R/SLRV-C or SLRV-D/SLRV-C against antisera to SLRV-D or SLRV-R. However, weak spurs were produced when SLRV-D or SLRV-R in wells adjacant to SLRV-C was reacted against antiserum to SLRV-C. This suggested that SLRV-D and R were closely related serologically but that these isolates were less closely related to SLRV-C. These affinities were also suggested by cross reactivities. The isolates R and D cross reacted to a similar extent with each others antiserum and to antiserum to SLRV-C. In the reciprocal reactions SLRV-C reacted to a lower extent with the heterologous antisera. The cross reacting antigens, however, were only separated in titre by one or two dilution steps suggesting that all antigens were closely related.

These results suggested all three isolates were closely related serologically, isolates R and D may have been identical serologicaly but further tests would be needed to confirm this.

As a technique gel-diffusion was apparently less sensitive at detecting SLRV than infectivity assay on *C. quinoa* . In comparative tests, the DEP of crude sap from infected *C. quinoa* in gel-diffusion was between 10^{-1}-10^{-2} while, infectivity end point of the same sap in *C. quinoa* was between 10^{-2} - 10^{-3}. Even with the purified

virus antigen, the DEP in gel-diffusion was only 10^{-2}-10^{-3} whereas the infectivity end point was between 10^{-3}- 10^{-4}.

4.4 Immunoelectrophoresis of SLRV-D, SLRV-R and SLRV-C

Immunoelectrophoretic techniques are one of the most powerful analytical tools for resolving antigens, as they differentiate between antigens on the basis of two independent criteria: electrophoretic mobility and antigenic specificity (Crowle, 1975 ; Van Regenmortel 1982). The antigen mixture is first separated into its components by electrophoresis in gels. Antiserum is then placed in a trough parallel to the path of electrophoretic migration and immunodiffusion precipitin lines are allowed to develop.As the different strains of a virus may vary in their overall net charge,electrophoretic mobility is often useful in distinguishing related strains (Ginoza and Atkinson, 1955; Walkey ***et al.***,1973; Hollings and Stone, 1975).

To characterise the three SLRV isolates (SLRV-D,R and C) further, the electrophoretic mobility of purified preparations of the viruses was examined in buffered agar-gel at pH 6.8 (Chapter 2.11c). The ***Caryopteris*** isolate (SLRV-C) was propagated in ***C. quinoa*** and purified using the butanol method as described for other two isolates.

After electrophoresis for 2h, the virus was located by placing homologous antisera in troughs parallel to the migration path as shown in **Figs 13a** and **13b**. The distance moved towards the cathode are given in **Table 22**.

Table 22 Results of electrophoretic mobility of SLRV isolates in buffered-gels

Virus	Electrophoretic movement (- mm)		
	Expt 1 (a)	Expt 2 (a)	Expt 3 (a)
D	3.1	NT	3.0
R	5.5	5.0	6.0
C	9.0	8.2	8.7

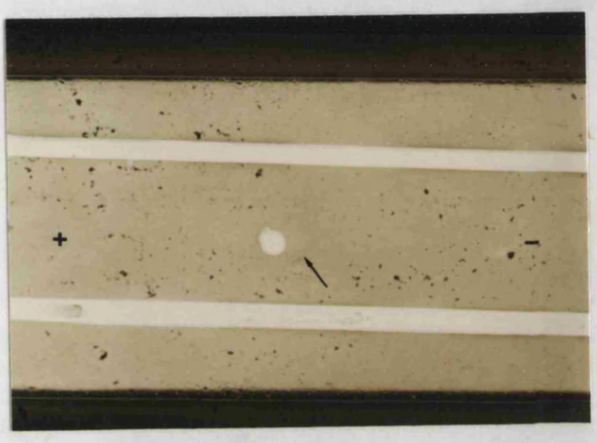
\- towards cathode
NT not tested
(a) mean of three replicates

The results showed that at the pH tested, the front of the precipitin arcs associated with parsnip isolates D and R were a shorter distance from the origin when compared to the ***Caryopteris*** isolate. This indicated that the ***Caryopteris*** isolate was electrophoretically distinct from the parsnip isolates, particularly SLRV-D.

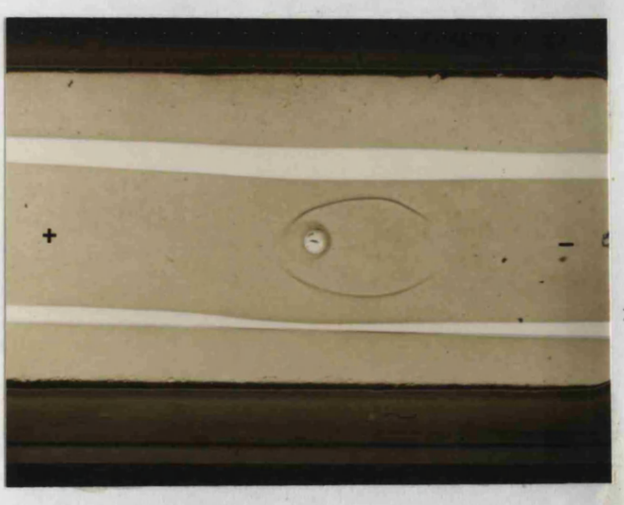
In these tests two electrophoretically distinct precipitin lines were sometimes formed with the parsnip isolates whereas, the ***Caryopteris*** isolate formed three distinct lines. The same preparations when reacted against their homologous antisera in agar gel-diffusion

Fig. 13 Immunolectrophoragram of SLRV isolates in buffered-agar gel, electrophoresed for 2h at 100v and developed with homologous antisera.
(a) SLRV-D (arrowed), (b) SLRV-C

(a)



(b)



tests produced either single lines (parsnip isolates) or two distinct lines (*Caryopteris* isolate). In a parallel experiment with purified preparations of healthy *C. quinoa* in immunoelectrophoresis, one of the lines associated with SLRV-C was identified as host protein.

4.5 Determination of molecular weight of capsid protein of SLRV-D and SLRV-R

Highly purified SLRV-D and SLRV-R preparations (after CPG chromatography) were dissociated by SDS treatment and electrophoresed on 7.5% polyacrylamide gels as described in Chapter (2.14).

Three clearly resolved bands were observed after staining with Coomassie Blue, but the top band was usually fainter than the other two. The results of this experiment are given in **Table 23** and **Fig.14.** The Mr of the SLRV isolates was determined by plotting a calibration line of known marker proteins (Log_{10} Mr)as a function of electrophoretic mobility **(Fig.15).**

According to these results the estimated relative molecular mass (Mr) of the three polypeptide bands of SLRV-D were 59,600, 42,000 and 30,000 (mean of six determinations). These results were comparable to that of Mayo *et al.,* (1974) who found 44,000 and 29,000 for the second and third bands respectively of isolates from strawberrry and raspberry. The first faint band with an

Fig 14 Electrophoretic mobility of SLRV-D and R coat proteins in 7.5% SDS-polyacrylamide gels (Electrophoresed for 3$_{1/2}$ h at 6 mA/gel). a, protein markers b, SLRV-D, c,SLRV-R

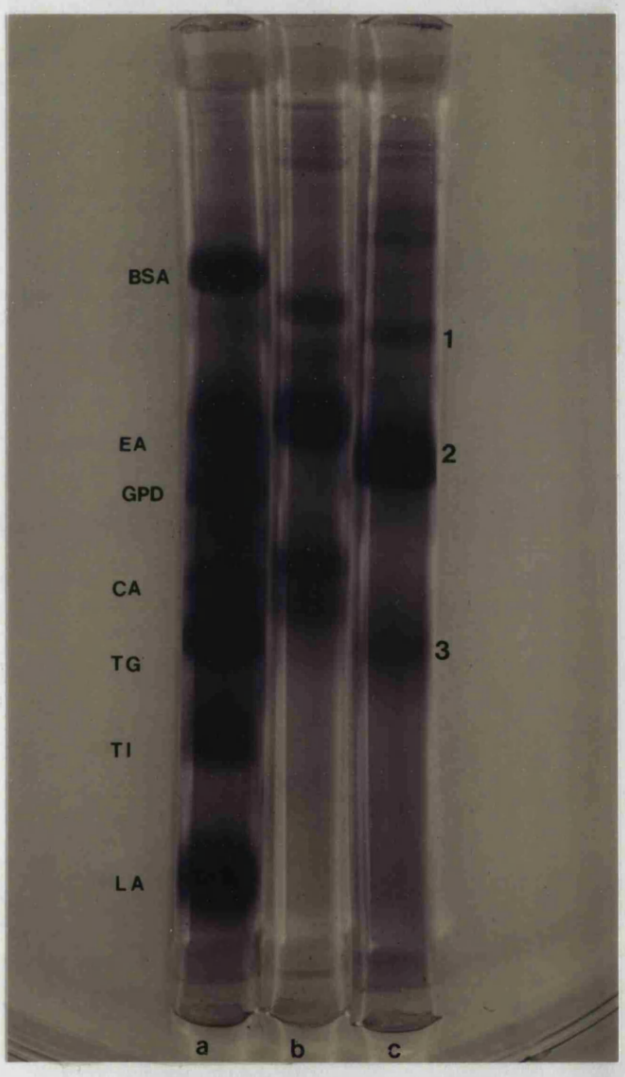
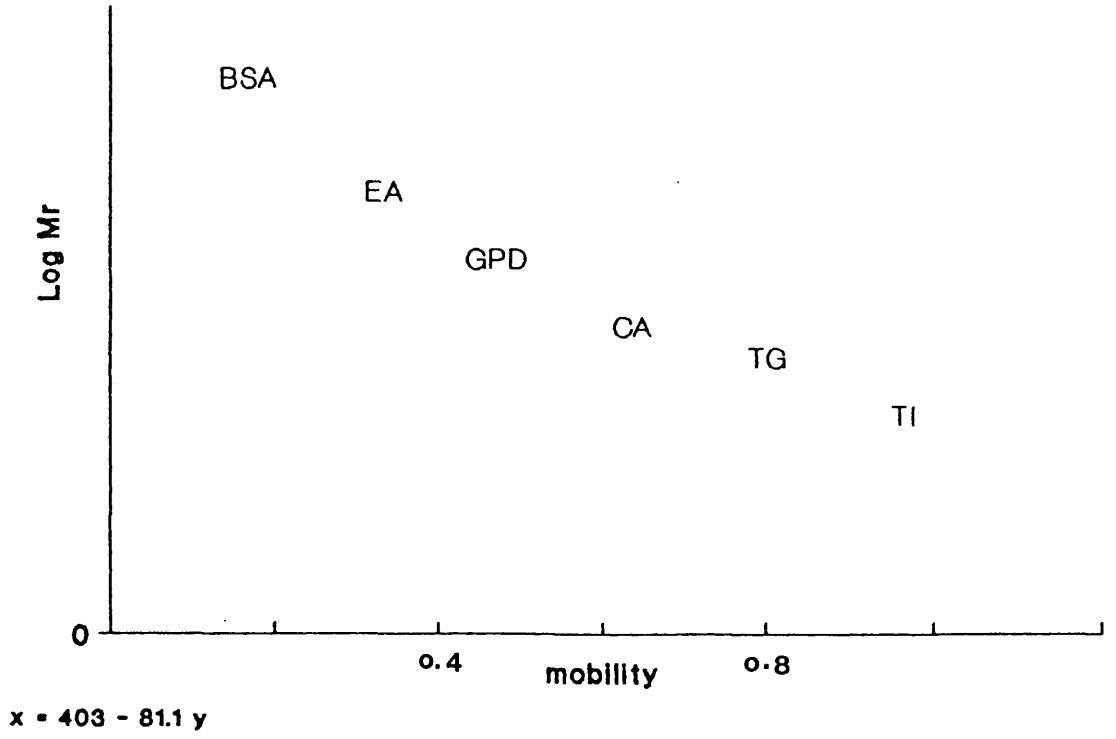


Fig. 15 . Estimation of Mr of capsid protein of SLRV



x = 403 - 81.1 y

estimated Mr of 59,000 may possibly be a dimer of the smaller polypeptide.

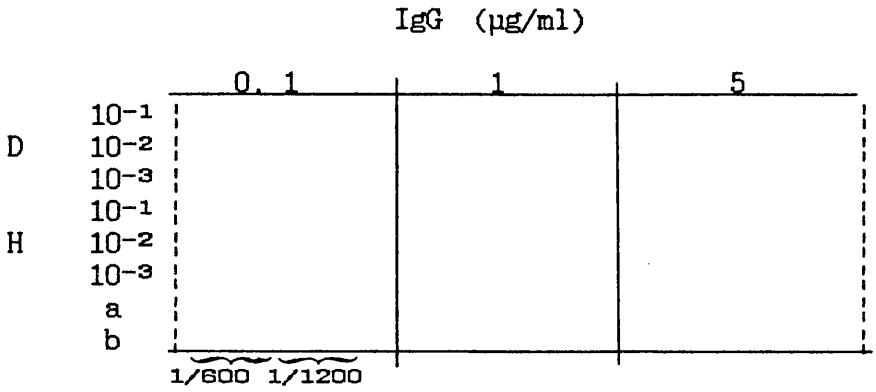
A comparison of the Mr of capsid proteins of SLRV-R and SLRV-D is given in **Table 23.** A slight variation in the Mr can be seen in the two isolates, but these were generally within the range of deviations (±10%) expected for the method (Weber and Osborn, 1969).

Table 23 A comparison of relative molecular mass of SLRV-D and SLRV-R capsid proteins

Virus	Relative molecular mass		
	band 1	band 2	band 3
SLRV-D	59.6±3.19	42.0±2.22	30.0±4.93
SLRV-R	60.9±4.67	45.0±1.83	32.5±3.50

4.6 Detection of SLRV isolates by DAS-ELISA

To detect SLRV-D and R by the ELISA technique described in Chapter (2.12), the optimum dilutions of reagents were determined first using a 'checkerboard' format as shown below.



a - Extraction buffer contol
b - Substrate control
D - infected samples
H - Healthy controls
IgG - Immunoglobulin

After carrying out a number of trial assays, the combinations were chosen to give maximum discrimination between the negative ('healthy controls') and the positive (infected) samples. These were 2 µg/ml coating antibody and 1/1200 dilution of detecting antibody for both SLRV isolates.

(a) Detection of SLRV-D,R and C using antiserum to SLRV-R in DAS-ELISA

Two uniform ***C. quinoa*** seedlings were inoculated with each isolates, using infective sap from ***C.quinoa*** (1:1/w:v). After inoculation the plants were kept in a growth cabinet at 18°C and systemically infected leaves were harvested 8 days later.

For each sample, 0.5g leaf tissue was homogenised in PBS-TPO (Chapter 2.12) and a series of five-fold dilutions prepared. The DEP in DAS-ELISA was

determined using 1/1000 dilution of detecting antibody to SLRV-R and the results are given in **Table 24.**

Table 24 DEP of different SLRV isolates detected by DAS-ELISA

SLRV isolate	DEP in DAS-ELISA (with antiserum to SLRV-R)
R	1/15,625+
C	1/15,625+
D	1/ 3,125 - 1/15,625

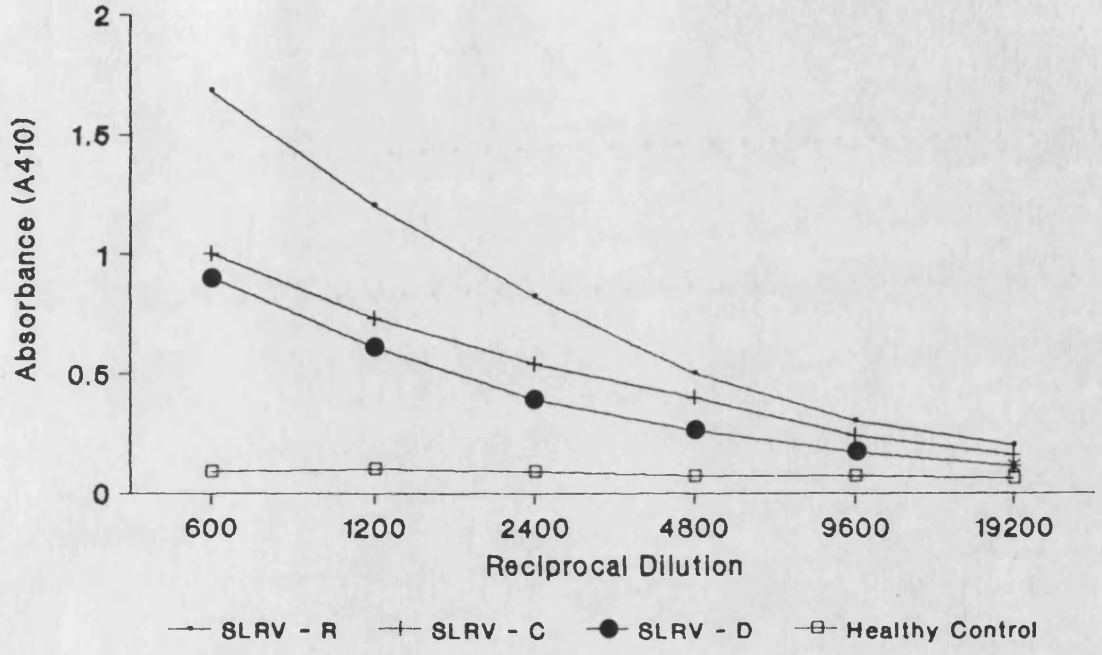
The results showed that a dilution of 1/1000 of antiserum R detected both homologous and heterologous isolates. It could not be determined from this experiment whether SLRV-R and SLRV-C occurred in higher concentration in *C. quinoa* than SLRV-D or whether the DEP was reduced by strain specificity.

In a further experiment, using a narrower range of antigen dilutions (two fold), the DEP was found to be 1/9,600 - 1/19,200 for SLRV-D and 1/19200+ for SLRV-R and C (**Fig. 16**). The results confirmed the high sensitivity of ELISA for virus detection compared to gel-diffusion and infectivity assay where DEP were 10^{-1} - 10^{-2} and 10^{-2}- 10^{-3} , respectively.

(b) Detection of SLRV-R, by DAS-ELISA in different hosts

Four different experimental hosts, *Chenopodium quinoa* , *C.amaranticolor, Nicotiana rustica*

Fig.16 ELISA readings of different SLRV isolates in *C.quinoa*



and cucumber were mechanically inoculated with SLRV-R. The concentration of the virus in each host was then estimated by the DEP of respective plant sap in DAS-ELISA.

All plants were inoculated at the seedling stages and grown in a glasshouse at 20°C. Systemically-infected leaves were harvested (0.5-1.0g) from each host ten days after inoculation (at this time systemic symptoms were not present in cucumber or ***N. rustica*** leaves which was a symptomless host of SLRV-R). The DEP in DAS-ELISA was detected using two-fold sap dilutions starting at 1/600. At least three replicates for each dilution were used and , because of this only two host species were tested in a single plate. Extracts of infected ***C.quinoa*** were used as a positive control at each time so samples tested on different plates could be compared.The results are shown in **Fig 17, 18** and **19.**

These results indicated that ten days after inoculation SLRV-R was present in higher concentrations in ***C. quinoa*** and ***C. amaranticolor,*** than in ***N. rustica*** and cucumber. Background absorbance levels of healthy extracts were generally low (< 0.1) although they were slightly higher with ***C.amaranticolor.***

(c) A comparison of SLRV concentration in root, leaf and petiole of parsnip infected with SLRV-D

An attempt was made to estimate the SLRV

Fig. 17 ELISA readings of SLRV-D in extracts of *C. quinoa* and *C.amaranticolor*

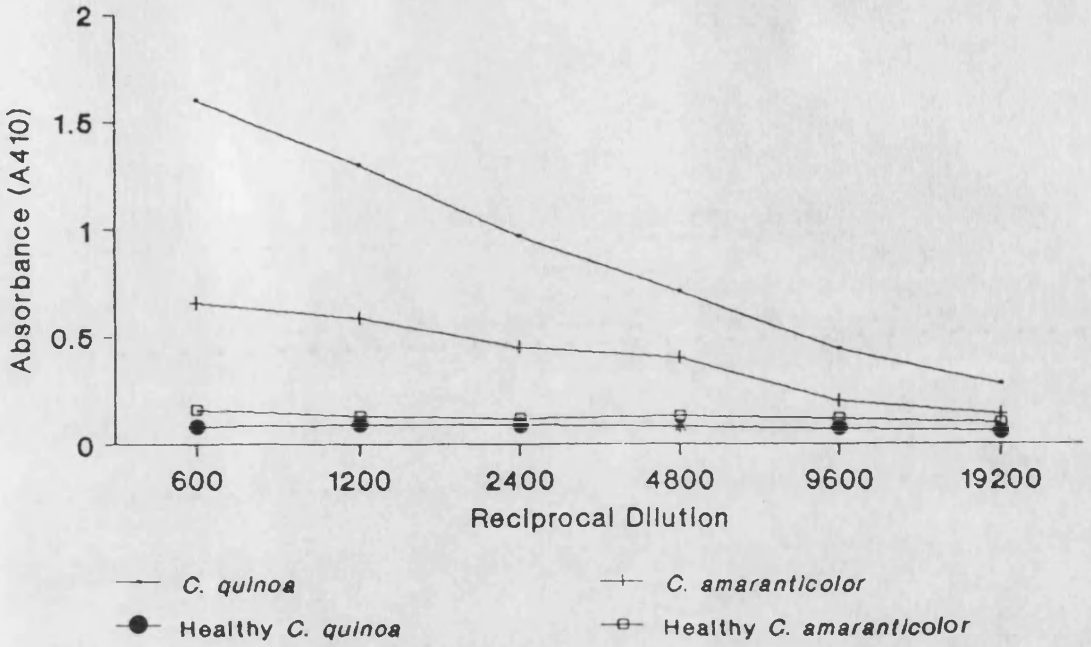


Fig. 18 ELISA readings of SLRV-D in extracts *of C.quinoa* and Cucumber

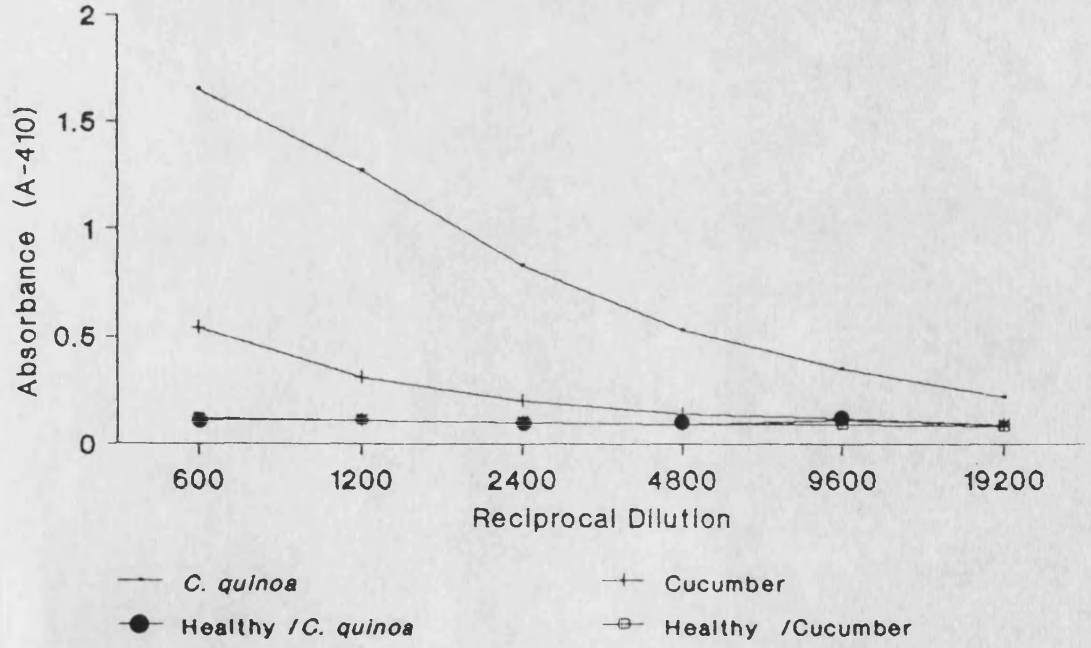
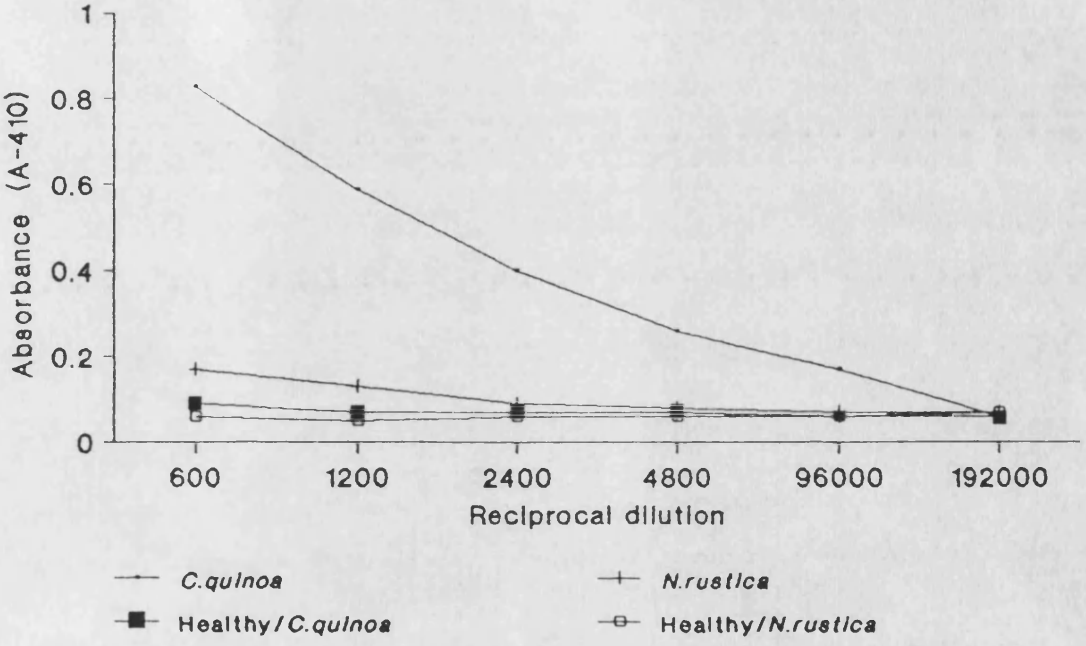


Fig. 19 ELISA readings of SLRV-D in extracts of *C. quinoa and N. rustica*



concentration in different parsnip tissues by DAS-ELISA. Tap root, leaf and petiole extracts were used from the same parsnip mother plant infected through seed, and the DEP determined using a series of five-fold dilutions. The experiment was done in two microtitre plates one with root and leaf tissues and the other with root and petiole tissues. The results are given in **Figs 20** and **21**.

The results showed that the DEP of virus antigens in all tissues tested was between 1/125-1/625. However, consistently higher A_{410} values were found with root extracts at most dilutions which at 1/5 were about four times that of leaf samples at the same dilution. In contrast to root extracts, leaf extracts at 1/5 dilution showed reduced A_{410} values compared with 1/25 dilution. This suggested that low dilutions of parsnip leaf extracts contained substances that intereferred with binding of virus to the coating antibody. Similar problems have been noted for other plants, particularly woody hosts (Clark and Adams,1977). According to these results the concentration of SLRV-D in parsnip tissue appear to be highest in root tissue and least in leaf tissues.

(d) The distribution of SLRV-D along the petiole

The petiole tissue of SLRV-D infected parsnip was taken from apical,middle and distal regions and the relative virus concentration was estimated by determining the DEP in DAS-ELISA using detecting antibody

Fig. 20 ELISA readings of SLRV-D in extracts of parsnip root and leaf

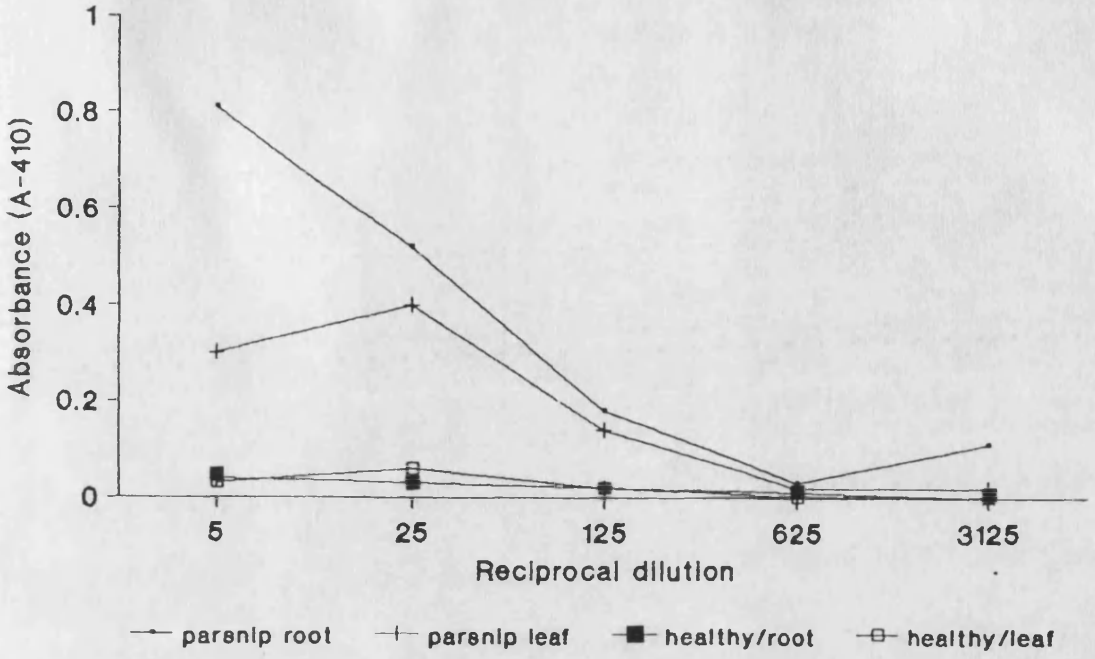
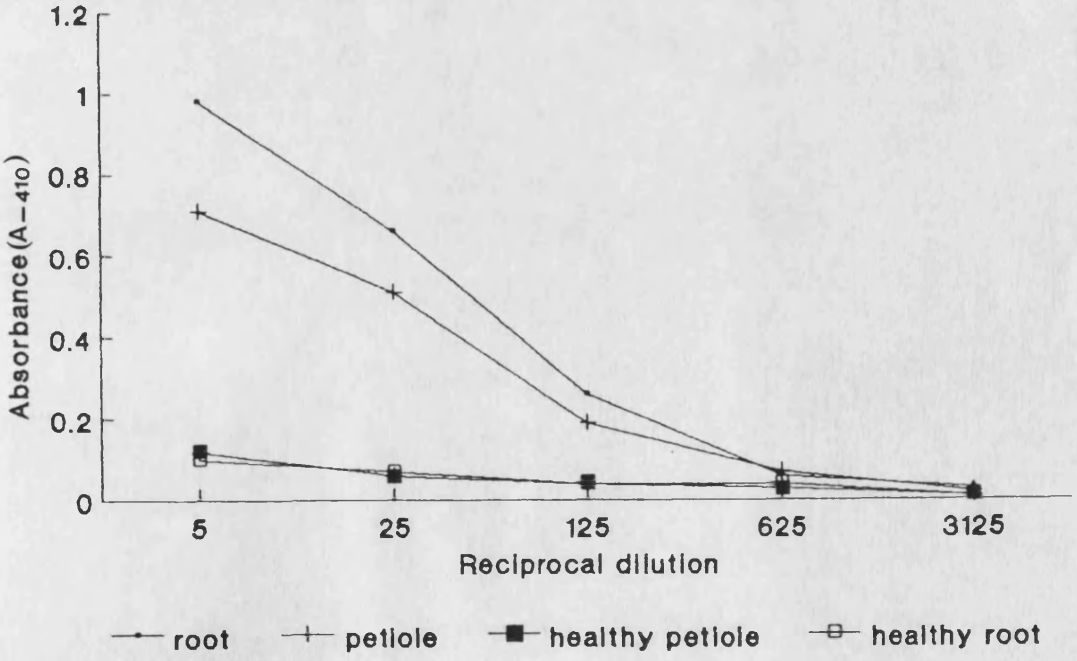


Fig.21 ELISA readings of SLRV-D extracted from parsnip root and petiole



to SLRV-D. The results are given in **Fig. 22.**

The results showed that the concentration of SLRV increased towards the distal region of the petiole.

4.7 Extraction and analysis of dsRNA from infected plants

4.7a Minimum quantity of diseased material needed for detectable dsRNA bands on polyacrylamide gels

Chenopodium quinoa leaves infected with SLRV-D were harvested 10 days after inoculation and used fresh in groups of five, ten, fifteen and twenty for dsRNA isolation (Chapter 2.13).

The dsRNA pellets from each tissue weight were dissolved in 80 ul of electrophoresis buffer containing 15% glycerol and electrophoresed for 4h at 5 mA /tube.

The dsRNA bands from all samples could be detected after staining the gels in ethidium bromide for 15 min (Chapter 2.13) although the 5g sample produced faint bands. In all samples three clear major dsRNA bands were readily detected, with the third species being brighter than the other two. In addition, three minor bands were also detected (**Fig. 23**). The identity of dsRNA bands was confirmed by DNAse and RNAse (high and low salt) treatment Chapter (2.13).

Fig.22 Distribution of SLRV-D in parsnip petiole estimated by ELISA (DEP)

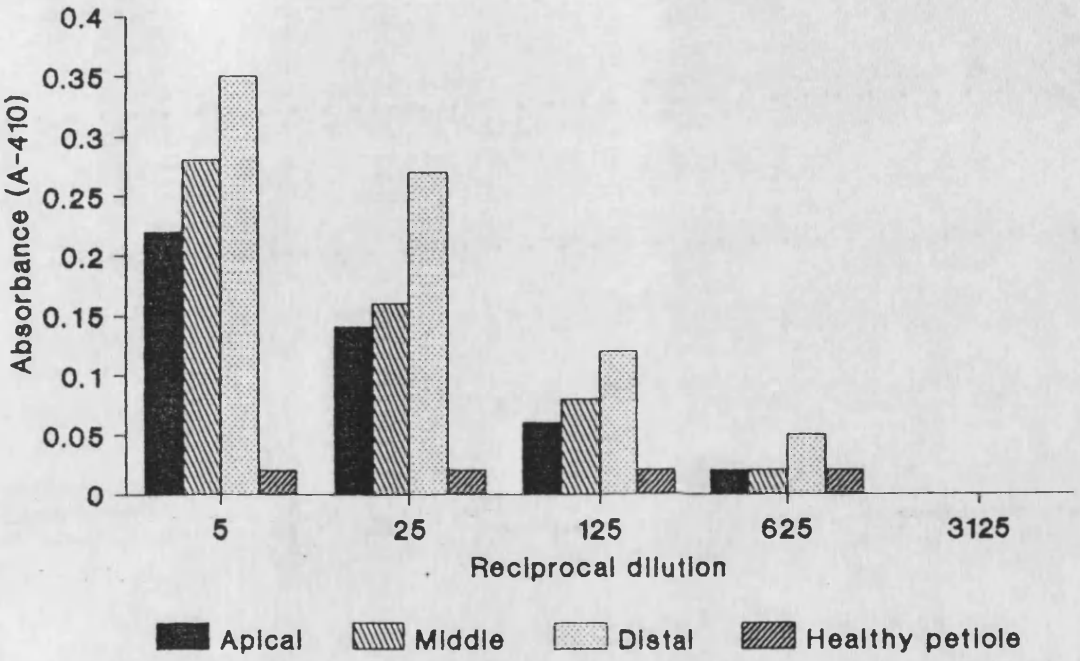
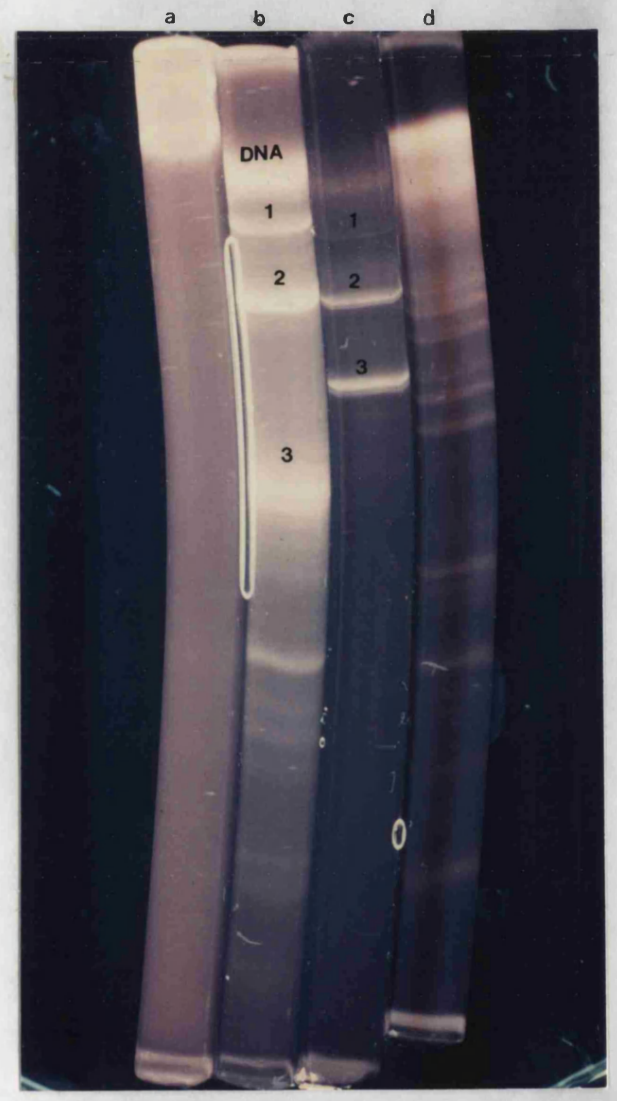


Fig. 23 Double-stranded RNA of SLRV-D extracted from *C. quinoa* and parsnip root, electrophoresed for 3h 45 min at 6mA/tube . (a) healthy (b) *C.quinoa* (c) parsnip root (d) Lambda digest



Estimation of relative molecular mass of dsRNA bands

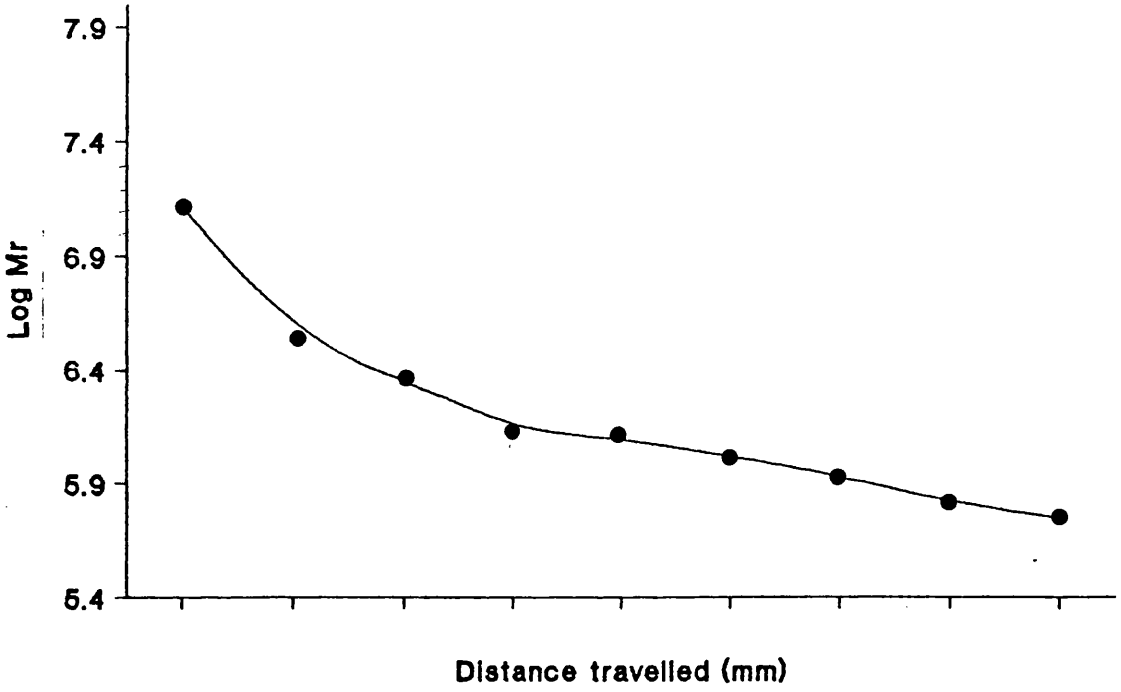
The relative molecular masses (Mr) were estimated using a calibration curve obtained for molecular weight markers by digestion of lambda DNA with,ECoR1/Hind 111 endonucleases (**Fig 24**). The first two bands were identified as the dsRNA form of genomic RNA, and had estimated Mr (ssRNA) 2.6 $x10^6$ and $1.2x10^6$ respectively. The Mr (ssRNA) of the third band was about 0.36-0.38 $x10^6$, slightly lower than the 'satellite' species of dsRNA reported for some isolates of SLRV (Mayo ***et al*** .,1974; Gallitelli ***et al*** .,1983).

4.7b Pattern of dsRNA extracted from different hosts

Attempts were made to extract dsRNA from systemically-infected leaves (10g) of ***C.quinoa*** , ***C. amaranticolor*** , ***N. tabacum*** cvs. 'White Burley' and 'Xanthi', and ***N. clevelandii*** inoculated at the 4 - 6 leaf stage with SLRV-D. The plants were randomised in a glasshouse at 20-24°C and leaves of ***Chenopodium spp.*** were harvested when the symptoms appeared (usually 7-10 days after inoculation) ***Nicotiana*** spp. (usually symptomless) were harvested 14 days after inoculation. In addition, dsRNA was extracted from leaf and root tissue of parsnip plants infected through seeds.

The results (**Figs. 25a** and **b)** showed that the dsRNA of SLRV-D could be readily extracted from all hosts tested. No differences were found in the basic

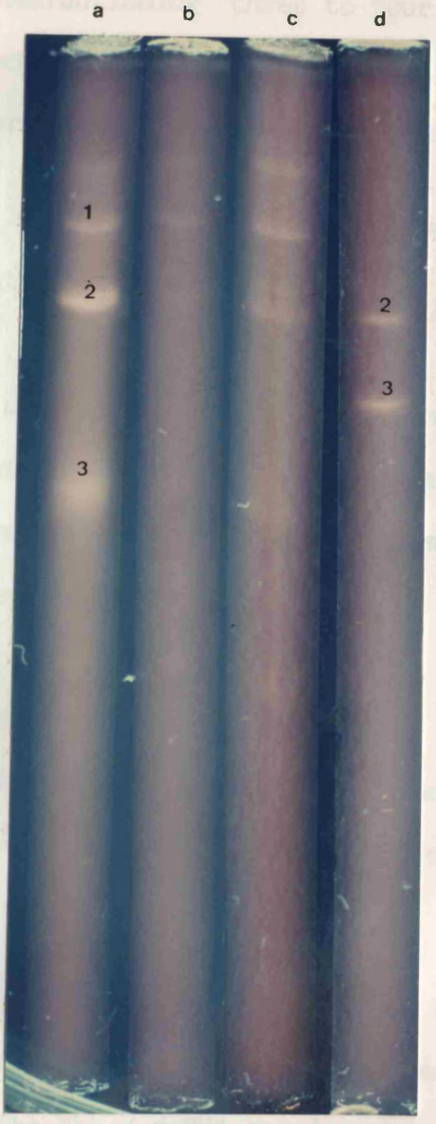
Fig.24 Estimation of Mr of dsRNA
of SLRV-D



$x = 87.7 - 11.5y + 0.011\ x^2$

Fig. 25a Double-stranded RNA of SLRV-D extracted from different host. Electrophoresed for 3h 45 min. From left to right *C. quinoa*, *C. amaranticolor* (SLRV-R), *N. clevelandii*, *N. tabacum* 'White Burley', parsnip root and *N. tabacum* 'Xanthi', lambda. Major bands numbered.

Fig. 25b DsRNA from SLRV-infected *C. quinoa* (a) *Nicotiana* spp. (b,c) parsnip (d); after incubation of gels in DNase and high salt RNase.

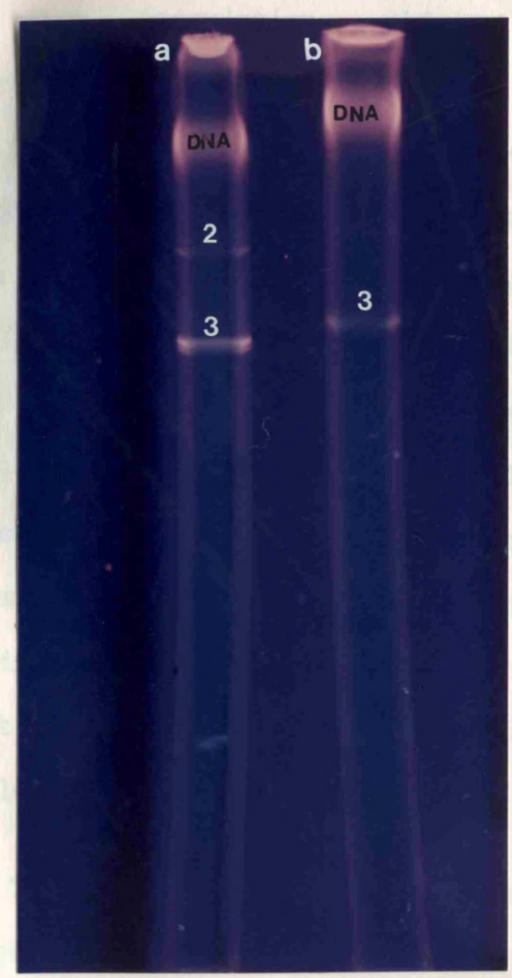


pattern of three major dsRNA species between *Chenopodium* spp. and *Nicotiana* spp. However, the number of minor bands and their intensity differed for the two groups. In *C.quinoa* and *C. amaranticolor* three to four minor bands were often detected, but in *Nicotiana* spp. these were mostly absent, or present in low concentrations.

In contrast, minor bands were never observed in preparations from the root or leaf tissues of parsnips (**Fig.26**). The three major dsRNA bands were readily detected in these preparations. The relative intensities of the dsRNA bands extracted from leaf and root tissues (similar weights) of a single parsnip mother plants were different. The dsRNA isolated from leaf tissues were generally much fainter (**Fig. 26**) and the first band especially was often at the limits of detection (for ethidium bromide staining). These results suggested higher levels of dsRNA and, therefore, virus replication in root rather than leaf tissues.

No differences in the estimated Mr of the genomic RNA of SLRV extracted from different plants species were found. The third dsRNA species extracted from parsnip (both leaf and root), however, which had a Mr of 0.40-0.45 (ssRNA) similar to the published data for SLRV satellite (Mayo ***et al,*** 1974 ; Galitelli ***et al.***, 1983) but slightly higher than the dsRNA 3 of all other hosts tested (0.36-0.38).

Fig. 26 Double stranded-RNA extracted form infected parsnip plant (a) root (b) leaf



4.7c Effect of post-inoculation temperature on the quantity of dsRNA in *C.quinoa* infected with SLRV-R

In previous experiments (Chapter 3.8) it was found that *C.quinoa* plants kept continuously at 26°C immediately after inoculation, did not show any differences either in symptom expression, or in the infectivity of their extracts when compared to those kept at lower temperatures (18°C). A comparison of dsRNA concentrations was made between manually inoculated plants grown at 26°C or at 18°C after inoculation. Uniform plants of the same age were used for both treatments and the strength of inocula, and environmental parameters (other than temperature) kept constant for both sets of plants. Systemically-infected leaves were harvested at intervals of six, nine, and twelve days after inoculation from each set of plants and dsRNA extracted separately from each sample.

The dsRNA pellets were dissolved in 100 ul of electrophoresis buffer and a hundred-fold dilution of this solution used to obtain the UV absorbtion spectra; the remaining samples were electrophoresed as before.

The concentration of dsRNA was estimated (Chapter 2.13) assuming an extinction coefficient of 20 for dsRNA of SLRV. The results are shown in **Table 25** and **Fig. 27** and **28**.

Fig. 27 Double stranded-RNA extracted from *C.quinoa* grown at 18°and 26°C after inoculation (diluted to 1:100). Leaves harvested after six days.

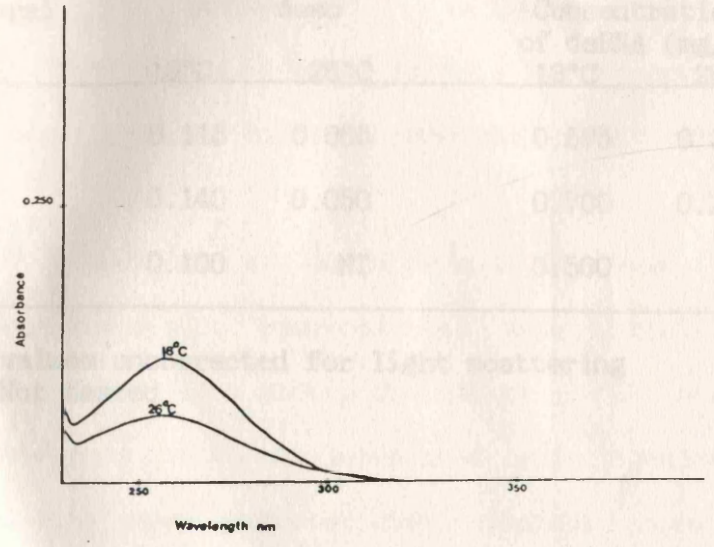


Fig. 28 Double stranded-RNA extracted from *C. quinoa* grown at 18°and 26°C after inoculation (diluted to 1:100). Leaves harvested after nine days.

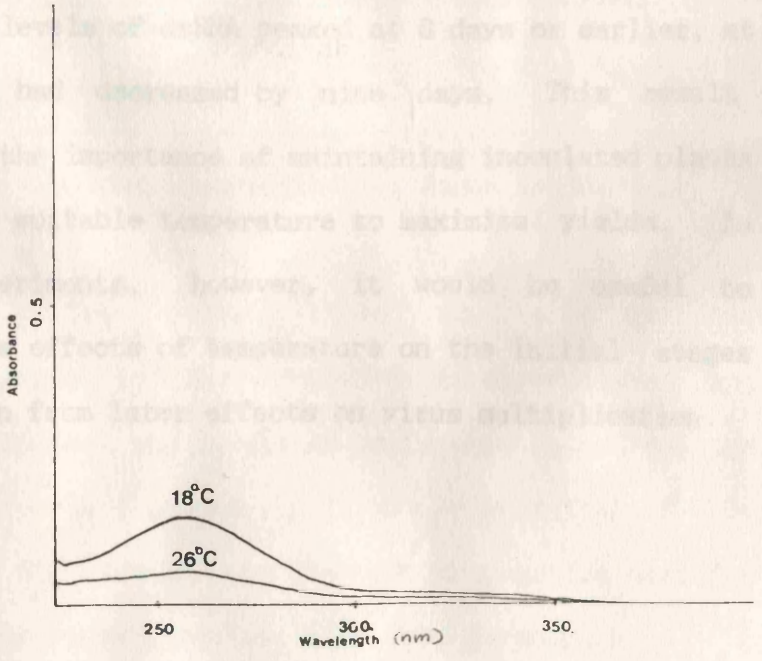


Table 27 Concentration of dsRNA extracted from *C. quinoa* grown at different temperatures after inoculation with SLRV.

Harvesting times (days)	(a) A_{260}		(a) Concentration of dsRNA (mg/ml)	
	18°C	26°C	18°C	26°C
6	0.115	0.065	0.575	0.375
9	0.140	0.050	0.700	0.250
12	0.100	NT	0.500	NT

(a) values uncorrected for light scattering
NT Not tested

The results showed that the plants exposed to 26°C after inoculation contained a lower amount of dsRNA when compared to plants grown at 18°C. The levels of dsRNA at 18°C rose to a peak at 9 days (when symptoms first appeared) and then decreased by 12 days. In contrast, levels of dsRNA peaked at 6 days or earlier, at 26°C, but had decreased by nine days. This result emphasises the importance of maintaining inoculated plants at the most suitable temperature to maximise yields. In future experiments, however, it would be useful to separate the effects of temperature on the initial stages of infection from later effects on virus multiplication.

4.7d A quantitative evaluation of dsRNA extracted from *C.quinoa* and cucumber infected with SLRV-R

To compare the amounts of dsRNA in different hosts, *C. quinoa* and *C. sativus* 'Marketer' infected with SLRV-R were selected as this isolate readily produced clear local and systemic symptoms in both these hosts.

Seedlings of cucumber and *C.quinoa* (ten days and four weeks old, respectively) were inoculated with SLRV-R and kept in a growth cabinet at 18°C until symptoms developed. Both non-inoculated and inoculated leaves/cotyledons were harvested when symptoms appeared and dsRNA was extracted from 10g of tissue. The results are shown in **Table 26** and **Fig. 29**.

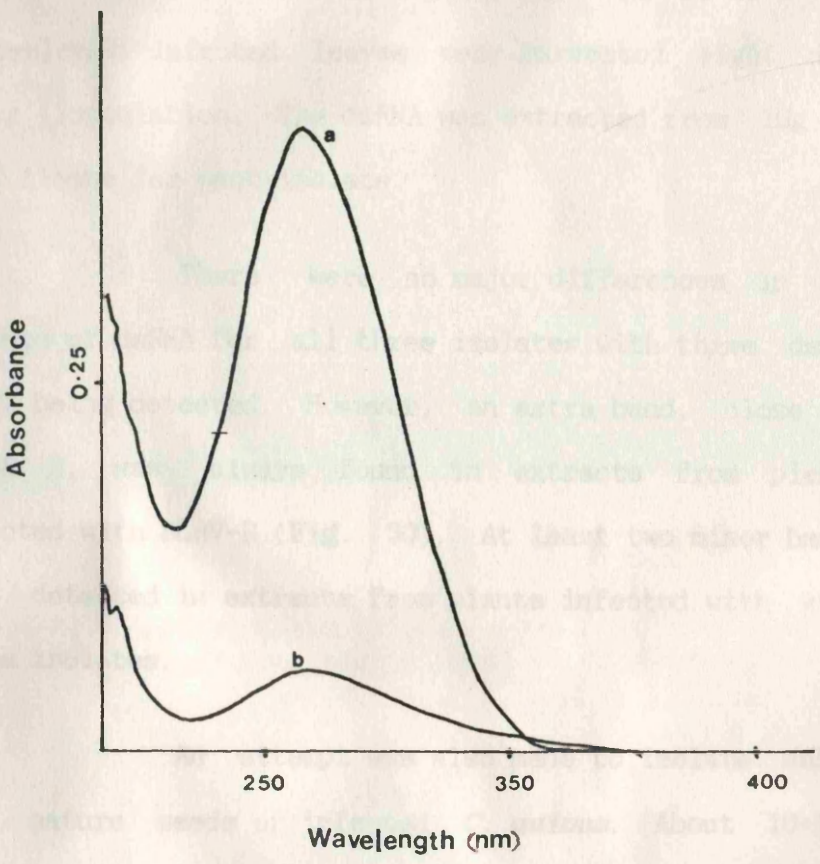
Table 26 Concentration of dsRNA extracted from two different hosts infected with SLRV-R

Host	Concentration of dsRNA (mg/ml) (a)
C.quinoa	2.1
cucumber	0.25

(a) values uncorrected for light scattering

The results showed that although the cucumber plants infected by SLRV-R produced local and systemic symptoms, the levels of dsRNA were relatively low when compared to *C.quinoa* . It was evident that of the two hosts, *C. quinoa* was the best propagation host for SLRV-R and probably other isolates from parsnips.

Fig. 29 Absorption spectra of dsRNA of SLRV-R extracted from *C. quinoa* and *Cucumis sativus* cv.Marketer (diluted 1/100). Leaves harvested 9 days after inoculation.



a, C. quinoa

b. Cucumber

4.7e A comparison of the dsRNA of two parsnip isolates and a *Caryopteris* isolate of SLRV

The isolates SLRV-D, R and C in *C.quinoa* were inoculated to uniform *C. quinoa* plants. Inoculated plants were randomised in a glasshouse at 20-25°C and systemically-infected leaves were harvested eight days after inoculation. The dsRNA was extracted from 10g of leaf tissue for each isolate.

There were no major differences in the pattern of dsRNA for all three isolates with three dsRNA bands being detected. However, an extra band, close to dsRNA 2, was always found in extracts from plants infected with SLRV-R (**Fig. 30**). At least two minor bands were detected in extracts from plants infected with each three isolates.

An attempt was also made to isolate dsRNA from mature seeds of infected *C. quinoa*. About 10-15g samples of fresh, mature seeds (before drying) were used to extract dsRNA. Only one major band corresponding to dsRNA-2 (**Fig 31**) was observed. If the other bands (dsRNA 1, satellite and subgenomic dsRNA) were present, they were below the limits of detectability. In the nepoviruses tomato black ring virus and raspberry ringspot virus, genetic determinants for seed transmissibility are carried on RNA-1 (Murant, 1981).

Fig. 30 Double-stranded RNA of three SLRV isolates extracted from *C. quinoa* . (a) healthy, (b) SLRV-D and (c) SLRV-C (d,e) SLRV-R note the duplicate bands of dsRNA-2 (f) Lambda digest

Fig. 31 Double-stranded RNA extracted from parsnip root (a) and from mature seeds of *C. quinoa* (b).

CHAPTER 5 Back-inoculation of SLRV to parsnip

5.1a Transmission by mechanical inoculation

Although it was not difficult to sap inoculate SLRV from parsnip leaf extracts to *C. quinoa*, attempts to infect parsnip with SLRV from infected *C. quinoa*, or from any other donor plant, were not successful. Inoculations were repeated many times using infected leaf extracts from a range of test plants. Because viruses are often readily transmitted between the same species (Bawden, 1954; Fulton, 1964) attempts were made to infect apparently healthy parsnips with leaf, root or seed extracts from parsnips infected with SLRV-D. In addition, partially-purified preparations of SLRV-D from *C. quinoa* were used.

Healthy parsnip seedlings were raised using seed collected from SLRV-free mother plants, cv. White Gem. However, when the first true leaf had emerged all plants were back-tested for SLRV on seedlings of *C. quinoa* to ensure that seedlings were free of SLRV before mechanical inoculation. Ten parsnip seedlings at the two to three leaf stage were inoculated with each inoculum source prepared in phosphate pH 7.8 buffer (Chapter 2.3) and kept at 18°C. Each parsnip seedling was back-tested on two *C. quinoa* seedlings at weekly intervals starting the first week after inoculation.

The results (Table 27) showed that parsnips were readily infected by infected root extracts but not by any other inocula tested.

Table 27 Mechanical inoculation of parsnip cv. White Gem with different sources of SLRV-D

Source of inoculum	Infection			
	Week 1	week 2	week 3	Week 6
C.quinoa	-	-	-	-
N.clevelandii	-	-	-	-
Parsnip (leaf)	-	-	-	-
Parsnip (root)	-	-	7/10(a)	7/10(a)
Parsnip (seed)	-	-	-	-
Partially purified SLRV from *C.quinoa*	-	-	-	-

(a) No. infected / No. inoculated
\- No infection

The results also showed that even with parsnip tap root extracts, it took a relatively long time for virus to be detected.

In an earlier experiment (Chapter 3.5) it was found that the susceptibility of cucumber plants to SLRV was increased by 30% when the plants were chilled for 4h prior to inoculation, compared to those which had not been pre-chilled. This test was repeated with pre-chilled parsnip which were inoculated with SLRV-D infected root

or leaf extracts of parsnip. This pre-chilling, however, did not increase the susceptibility of parsnips to SLRV infection (**Table 28**). The reason may be that parsnips are not a 'chill sensitive' species like cucumber. As in the previous experiment only the root extract was infective.

Table 28 The effect of pre-chilling of parsnip on its susceptibility to infection by SLRV-D

Treatment	Infection(a)
Pre-chilled for 4h after 24h dark treatment	7/10
No pre-chilling after 24h dark treatment	8/10

(a) No. infected / No. inoculated

The failure to infect parsnips by leaf inoculum suggested that leaf sap may contain a low virus concentration compared to that in root extracts. This fact was also supported by results of dsRNA analysis and ELISA tests (Chapter 4.7C and 4.6)with leaf and root extracts of parsnips. Alternatively, virus inhibitors may have been present in leaf sap. Experiments were done, therefore, to compare the concentrations of virus and inhibitors in these two tissues.

The virus infectivity was estimated from the DEP of root or leaf extracts on *C. quinoa* seedlings. A series of ten-fold dilutions of leaf or root sap were made from the parsnip mother plant infected with SLRV-D

through seed, and the DEP determined (Chapter 2.9)

Table 29 DEP of SLRV-D infected parsnip leaf and root sap on *C. quinoa*

Source of inoculum	DEP in *C. quinoa*
Leaf sap	10^{-1} - 10^{-2}
Root sap	10^{-2} - 10^{-3}

The results (**Table 29**) indicated a relatively higher virus concentration in infected root sap than in leaves, or alternatively, a lower concentration of inhibitors. Furthermore, within six days buffered root inoculum (1:1 / w:v) produced in *C. quinoa* about 175-250 local chlorotic lesions (**Fig. 32**), followed by systemic chlorosis and apical necrosis, while no local lesions were produced with buffered leaf inoculum (1;1 / w;v) although mild systemic symptoms developed later. This also suggested that there might be an inhibitor in leaf extracts. A leaf scorch was observed (**Fig. 33**) with leaf inoculum possibly due to some toxic component of leaf sap.

The presence of inhibitors in the tap root and leaf sap was investigated by mixing tissue sap from a healthy parsnip plant with an inoculum of tobacco necrosis virus (TNV,isolated from a soil sample during 'bait' tests for nematodes), and observing the number of necrotic local lesion produced on *Phaseolus vulgaris* cv. Prince.

Fig. 32 Chlorotic local lesions induced by SLRV-D infected parsnip root sap on *C. quinoa*

Fig. 33 Leaf scorch induced by SLRV-infected parsnip leaf sap on *C.quinoa*

Tobacco necrosis virus inoculum was prepared by grinding infected ***P. vulgaris*** leaves in 0.05 M phosphate buffer, pH 7.8 at 1:30 (w : v) a dilution which in preliminary tests produced easily countable numbers of lesions. Leaf and root sap from the same healthy parsnip mother plant were prepared in the same buffer, at 1 : 2 (w : v). All extracts were expressed through muslin, and equal volumes of TNV and parsnip tissue extract were mixed and, assayed on six half leaves of ***P. vulgaris*** (Chapter 2.5). Lesions were counted five days after inoculation and the results are given in **Table 30.**

Table 30 Effect of parsnip extracts on the infectivity of TNV on ***P. vulgaris***

Treatment	Mean lesion no. per half leaf
A, TNV+Parsnip leaf sap	65(a)
B, TNV+Parsnip root sap	141
C, TNV+Phosphate buffer	244

(a) Significantly different from C ($P < 0.05$, 5% L.S.D = 47.69)

The results showed that parsnip leaf sap had a significant ($P < 0.05$) inhibitory effect on the infectivity of TNV with a percentage inhibition of about 73% while with root sap, inhibition was 42%.

The pH of leaf and root sap of parsnip extracted in equal volumes of phosphate buffer (pH 7.8) were found to be 7.0 and 7.5, respectively (6.0 and 6.5 respectively when extracted in tap water). Some part of the inhibitory effects therefore, may have been related to changes of pH, especially with leaf extracts.

These results suggested, however, that the presence of sap inhibitors as well as relatively low virus concentrations (**Table 29**) contributed towards the failure of leaf inoculum to infect parsnip.

The reason for the failure to infect parsnip with other sources of inocula including partially-purified preparations is not known. Even with root sap however, two to three weeks were needed for infection to be detected compared with other hosts (six to eight days). This suggested that there may be a restricted movement or multiplication of virus between or within parsnip leaf cells.

5.1b The effect of different post-inoculation temperature on SLRV infection of parsnip by mechanical inoculation.

Healthy parsnip seedlings (previously indexed on ***C. quinoa***) were given a 24h dark treatment and inoculated with infected root extracts of SLRV-D at 1 : 0.5 (w:v). Inoculated plants were covered with damp newspaper for 20h at room temperature and then transferred

to growth cabinets at 18° or 26 ±2°C with 16h photoperiods. Ten plants were inoculated for each treatment and all plants were back tested on *C. quinoa* seedlings for symptomless infection four weeks after inoculation. The results are given in **Table 31.**

Table 31 The effect of post-inoculation temperature on the infection of SLRV-D in parsnips by mechanical inoculation.

Post-inoculation Treatment	Infection (a)
18 ±2°C	7/10
26 ±2°C	0/10

(a) No.infected / No.inoculated

These results suggested that the higher post-inoculation temperature (26°C) prevented the infection of parsnips by SLRV or at least reduced concentrations of SLRV to below detectable levels. In contrast, 70% of plants kept at 18°C after inoculation became infected with SLRV. With *C.quinoa* plants kept at 26°C after inoculation, all became infected and showed symptoms (Chapter 3.8). Similarly seven out of 12 cucumber seedlings were symptomlessly infected when kept at 26°C (Chapter 3, **Table 14**). Unlike these hosts, therefore, parsnip apparently becomes resistant to SLRV infection at moderate temperatures (26°C).

5.2a Transmission by nematodes

Apart from being seed transmitted, SLRV is also naturally spread by the nematode *X. diversicaudatum* Micoletsky (Lister, 1964). In this experiment, this vector was used to back-inoculate SLRV to parsnips.

X. diversicaudatum was isolated and identified by the structure of mouth parts, anterior gut region and posterior part (**Figs.34,35** and **36.**, Lamberti *et al.*, 1975).

Nematodes were assayed for infectivity as described in Chapter 2.7b. Nematodes free of SLRV were fed on young infected parsnip roots with SLRV-D, for about six weeks. Infected plants were removed, the soil sieved and mixed well and three healthy parsnip seedlings per pot (three to four leaf stage) were planted in soil in 10 cm pots (Chapter 2.7b). Healthy parsnips were grown for six to ten weeks and leaves back tested on *C. quinoa* at weekly intervals. A control experiment was also done by allowing SLRV free nematodes to feed on healthy parsnips and then planting healthy parsnip seedlings in the soil as described above. Results are shown in **Table 32.**

Fig. 34 Anterior region of *X. diversicaudatum*

[X 100]

Fig. 35 The posterior part of *X. diversicaudatum* showing peg-like appendage (arrowed) characteristic of the species.

[X 100]

Fig. 36 Heat relaxed position of *X. diversicaudatum*

x 33

Table 32 Nematode transmission of SLRV-D from infected parsnip to healthy parsnips and *C. quinoa*

Pot No.	Test plant	Infection(b) wk1	wk2	wk3	wk4	wk5	wk6
1	Parsnip	-	-	-	-	1/3	1/3
2	"	-	-	-	-	-	1/3
3	"	-	-	-	-	-	2/3
4	"	-	-	-	-	-	2/3
5	"	-	-	-	-	-	1/3
6	Parsnip(a)	-	-	-	-	-	-
7	*C.quinoa*	-	-	-	-	-	6/6

(a) Controls- nematodes previously fed on healthy parsnips
(b) No.infected / No.introduced
\- no infection detected

The results showed that 100% (6/6) of *C. quinoa* and about 47% (7/15) of healthy parsnips became infected with SLRV-D by nematode transmission. After six weeks access period, SLRV could only be detected in the leaves of a single parsnip plant. In the sixth week, test plants were removed from soil and both leaves and roots were indexed separately in *C. quinoa*. It was found that although virus was detected in parsnip roots, of seven plants it could only be detected in the leaves of three or four plants. Possibly, there was a delay before the virus in roots was transported to the leaves, or if present in leaves had not reached high enough concentrations to be

detected.

The next experiment was tested the time required for healthy parsnip roots to become infected by nematode transmission.

The experiment was performed as before, but six healthy parsnips per pot (10 cm) were used to increase the availability of roots to the nematodes. A pot was randomly selected at weekly intervals (beginning the second week), and the roots were harvested and indexed on *C. quinoa* seedlings. The results are shown in **Table 33.**

Table 33 Time required to infect parsnip roots by SLRV-infected nematodes.

Pot	'Feeding' time (weeks)	Infection (a)
1	2	1/6
2	3	2/6
3	4	2/6
4	5	3/6
5	6	2/6
6	10	3/6

(a) No. infected / No. introduced

These results indicated that some parsnip roots became infected with SLRV within a relatively short exposure ('feeding') time. In further experiments, healthy parsnip roots become infected within 10 days, and in all

such experiments the mean percentage of parsnips that became infected never exceeded 55% (the range was within 45-55%). According to Das and Raski (1968), *X. index* could both acquire or inoculate virus even within fifteen minutes feeding time.

5.2b Effect of age of parsnips on SLRV infection by nematodes

It has been reported that the susceptibility of a host plant to viruses varies with its age, and vigorously growing young seedlings have generally been found to be more susceptible than older plants (Matthews, 1981). In this experiment the effect of age of parsnips on the infection of SLRV by nematodes was investigated.

Parsnip seedlings infected with SLRV-D were pricked into soil containing SLRV-free nematodes in 30x15x5 cm trays. After allowing nematodes access for six weeks the parsnips were removed. The soil in all trays was then thoroughly but gently mixed , and 300 ml samples put into two set of seven 10 cm pots.

Three parsnip plants with two to three true leaf stage were introduced to each pot to give a total of 21 plants (seven replicates). In another seven pots, seeds of healthy parsnips collected from SLRV-free mother plants were sown (6 - 8 seeds per pot) and on germination

three seedlings per pot were retained and the others discarded.

All pots were kept at 20 - 24°C in a glasshouse for four to five weeks and the roots were harvested and indexed by sap inoculation on *C. quinoa* .The results are shown in **Table 34.**

Table 34 Effect of parsnip age on SLRV infection by nematodes

Stages of parsnips	Infection (a)	% infection
2-3 true leaves	12/21	57
young seedlings	17/21	81

(a) No. infected / No. introduced

The results showed that newly germinated seedlings were more susceptible to infection by nematodes than the 5$_{1/2}$ weeks old plants. The infection rate in young seedlings was 24% higher than that of the older plants although this difference was not significant ($P>0.05$).

5.2c Effect of temperature on the infection of SLRV by nematodes

In a previous experiment (Section 5.1b) post-inoculation temperature had a highly significant effect on the infection of mechanically-inoculated parsnips by SLRV (**Table 31**). Experiments were done,

therefore, to see if the susceptibility of parsnip roots to nematode-borne infection was affected by changes in temperatures.

Two trays (15 x 10 cm) of soil containing SLRV-free nematodes were sown with parsnip seeds cv. White Gem infected with SLRV-D (estimated infection 80- 90%) and kept in a glasshouse at 20-24°C for germination. After six weeks the parsnips were removed and the soil sieved and thoroughly mixed. The soil (in 15 x 10 cm) trays was then planted with parsnip seeds collected from a SLRV-free mother plant. Trays were then kept either at 18°C or at 26°C in growth cabinets illuminated for 16 h/day.

Four weeks after germination the roots were harvested and assayed for SLRV by DAS-ELISA using homologous antiserum. About 60-80 plants were tested in groups of four and the proportion of infected plants was calculated (Chapter 2.12). The results are shown in **Table 35.**

Table 35 Effect of temperature on the transmission of SLRV to parsnip by nematode inoculation.

Temperature	No.of groups positive for SLRV in ELISA (a)	Estimated proportion infected
18°C	4/19	5.7
26°C	11/16	25.2

(a) No.of observed positive groups/No.of groups tested

The results showed that a higher proportion of plants became infected at 26°C than at 18°C.

The same experiment was repeated with *C.quinoa* as bait plants and infection was assessed by indexing groups of four plants, onto *C. quinoa* .

Table 36 Effect of temperature on the transmission of SLRV to *C. quinoa* by nematodes

Temperature at which test plant kept	Infection (a)	Estimated proportion infected
18°C	10/20	16
26°C	20/20	100

(a) No.of groups infected / No.of group tested

The results (**Table 36**) with *C. quinoa* were similar to those for parsnip but *C. quinoa* appeared to be more susceptible to nematode-borne infection than parsnip. As with parsnip a higher number of plants were infected at 26°C than at 18°C. This contrasted with the infection of *C. quinoa* and cucumber by mechanical inoculation where lower temperatures apparently increased susceptibility. Possibly the higher temperature increased the feeding activity of nematodes so that a relatively higher number of plants were infected at 26°C. The higher temperature may also have increased root growth and the availability of roots to nematodes. The soil temperature is normally at least 2 - 3 degrees lower than the air

temperature and may have been closer to the optimum for feeding, reported by Boag (1978) to be 20-25°C.

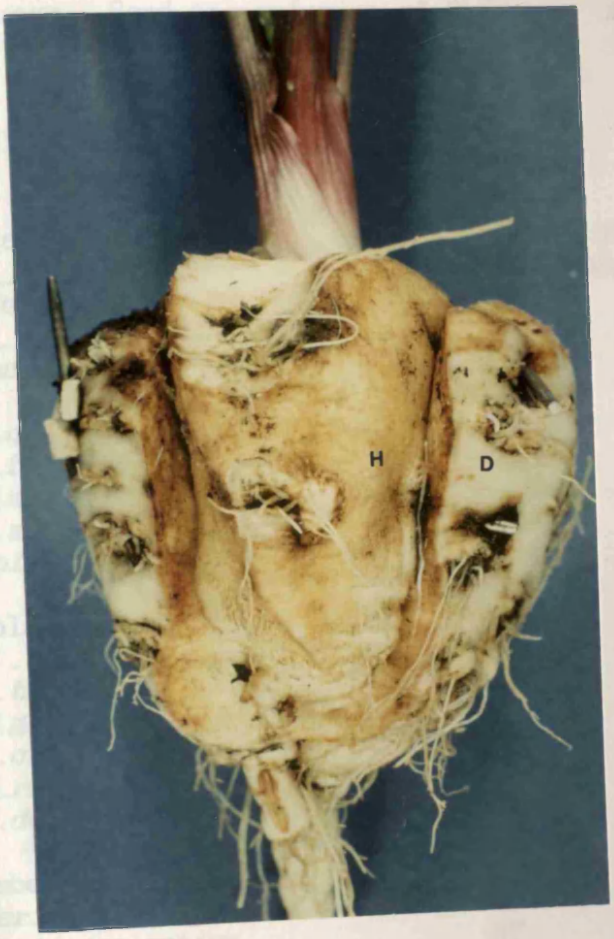
5.3 Transmission of SLRV-D by root grafting

It has been found in previous experiments (section 5.1) that SLRV can be returned to parsnip by mechanical inoculation with infected root extracts. In this experiment, an attempt was made to infect parsnip with SLRV by grafting using infected root as a tissue donor.

Healthy parsnip plants were obtained from seeds collected from an SLRV-free mother plant, and infected plants from seeds of the mother plant infected with SLRV. When the plants were three months old with tap roots about 2.5 cm in diameter, a saddle graft was made between healthy root (scion) and infected rootstock as described in Chapter 2.8. Leaves from healthy plants was assayed for virus at weekly intervals three weeks after grafting.

Six out of ten plants became infected with SLRV-D three months after root grafting. Graft transmission of viruses in nature, however, is probably uncommon; but may occur through chance grafting of roots as they grow together (Walkey, 1985).

Fig 37 An inverted saddle graft of healthy parsnip plant to SLRV-D infected root.



H, healthy root D, infected root

CHAPTER 6 Pollen and seed transmission of SLRV

6.1 Seed transmissibility of SLRV-D and SLRV-R

Seeds were collected from naturally-infected parsnips and several experimentally infected hosts (Chapter 2.6) and about 100-150 seeds sown in 30 x 15 cm trays. Seed transmission of SLRV was assessed by indexing 40 - 50 seedlings individually onto *C. quinoa*. The results are given in **Table 37.**

Table 37 Percentage seed transmission of SLRV

Host species	% Transmission SLRV-R	SLRV-D
Chenopodiaceae		
C.quinoa	98-100	98-100
C.foetium	100	100
C.album	100	100
C.amaranticolor	98-100	98-100
Spinacea oleracea	76	71
Solanaceae		
N .tabacum 'White Burley'	35	28
N.tabacum 'Xanthi'	85	75
N.glutinosa	50	49
N.clevelandii	98	96
N.rustica	72	68
N.debneyi	20	18
Umbelliferae		
Coriandrum sativum	-	86
Pastinaca sativa	NT	80
Cucurbitaceae		
Cucumis sativus 'Marketer'	98-100	80-90

NT not tested
\- no infection

The results showed that there was little difference in seed transmissibility between the SLRV

isolates tested. Both isolates were readily seed transmitted in *Chenopodium* spp. and cucumber where seed transmission often exceeded 80%. In *Nicotiana* species transmission varied from 18 - 98%, but both isolates were transmitted to similar levels in each species.

The effects of SLRV on vigour of the seedlings infected through seed varied with host species and the isolates. Seedlings of coriander, for example, infected with SLRV-I, showed a loss of vigour and severe necrosis of newly emerged leaves which distinguished them from non-infected seedlings. In contrast, the 'intermediate' isolate D caused no apparent loss of vigour or other symptoms,and, infected coriander seedlings could not be distinguished from healthy seedlings.

The 'mild' isolate R produced a slight mottling in infected seedlings of *N. clevelandii*, *S. oleracea*, *C. quinoa* and *C. album*, but there were no symptoms or apparent loss of vigour in any other host tested. All three parsnip isolates of SLRV produced a mild chlorotic mottle and a reduction in vigour in *C. quinoa* infected through seeds.

Parsnip seedlings grown at 18°C with about 6,000 lux illumination for 16 h / day and infected with SLRV-D, often developed mild chlorotic mottle and chlorotic spots and sometimes necrosis of young newly emerged leaves. If the plants at this stage were not re-

potted they developed a severe chlorotic mottle (**Fig.38**). However, when these plants were re-potted or treated with fertilizer while in the original pots, the mottling symptoms gradually disappeared. A small proportion (25%) of parsnip seedlings did not show symptoms but were found to be infected.

6.2 Effect of SLRV-D and SLRV-I on the germination of infected host seeds

Seeds collected from infected and healthy hosts were sown in 30 x 15 cm trays and germinated in a mist-house at 20 - 22°C. Three batches of 100 seeds from each species to be tested were sown, and the mean percentage germination determined. The rate of germination was determined over a one month period for spinach infected with SLRV-D and coriander infected with SLRV-D or SLRV-I.

The results (**Table 38**) showed that final percentage germination of SLRV-D infected spinach and coriander seeds was significantly lower ($P<0.05$) than that of healthy seeds. The rate of germination of infected seeds was also much lower than that of healthy seeds (**Figs.39a** and **39b**). Similarly, the percentage germination of coriander seeds infected with the 'severe' isolate (SLRV-I) was also significantly lower ($P<0.05$) than that of those infected with SLRV-D. The percentage germination of seeds from parsnip infected with SLRV-I and D was

Fig. 38 Chlorotic mottle associated with SLRV-D (infected through seeds) in parsnip leaves grown at 18°C for 3 1/2 months in 10 cm pots.

Fig. 39a Rate of germination of coriander seeds infected with SLRV-I and SLRV-D

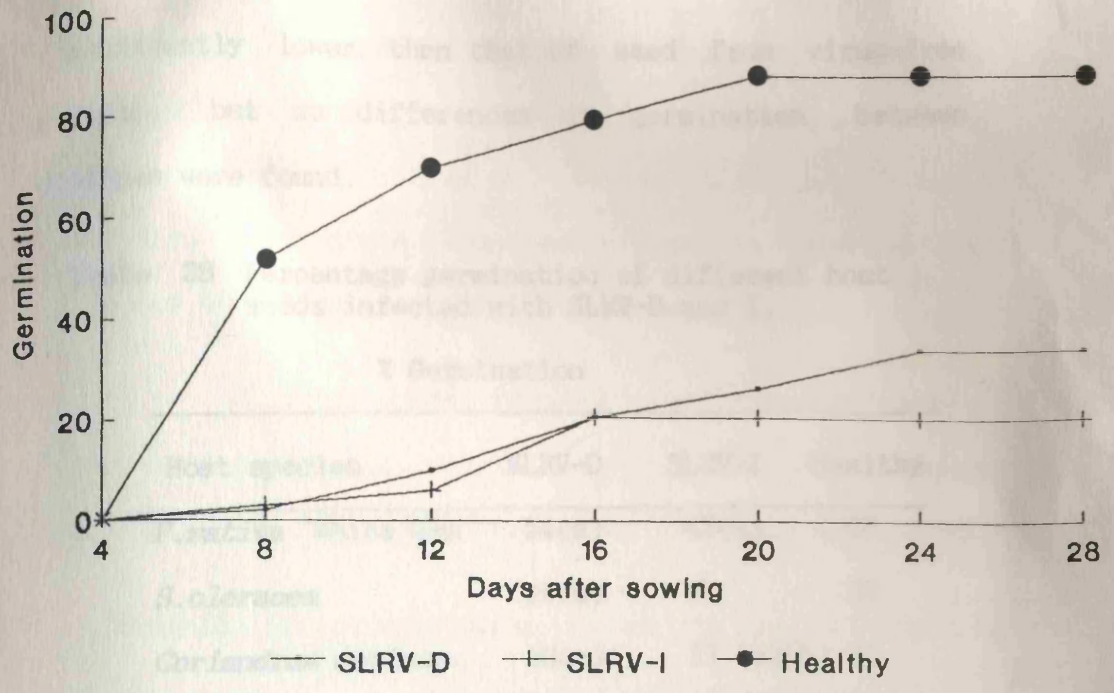
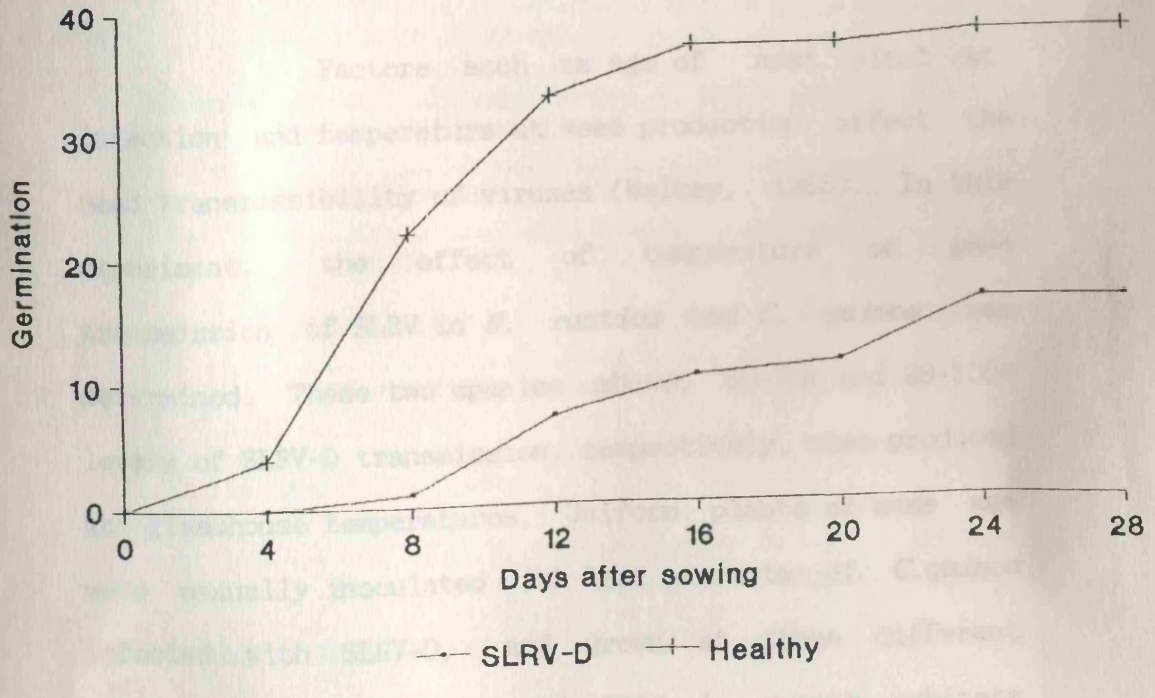


Fig.39b Rate of germination of spinach seeds infected with SLRV-D



significantly lower than that of seed from virus-free parsnip, but no differences in germination between isolates were found.

Table 38 Percentage germination of different host seeds infected with SLRV-D and I.

	% Germination		
Host species	SLRV-D	SLRV-I	Healthy
P.sativa 'White Gem'	54(a)	47(a)	88
S.oleracea	16(a)	NT	39
Coriandrum sativum	36(a)	17 (a)(b)	81

(a) Significantly different from healthy ($P<0.05$)
(b) Significantly different from SLRV-D ($P<0.05$)
NT Not tested

6.3 Effect of post-inoculation temperature on the seed transmission of SLRV-D

Factors such as age of host plant at infection and temperature at seed production affect the seed transmissibility of viruses (Walkey, 1985). In this experiment, the effect of temperature on seed transmission of SLRV in *N. rustica* and *C. quinoa* was determined. These two species showed 60-70% and 98-100% levels of SLRV-D transmission, respectively, when produced at glasshouse temperatures. Uniform plants of same age were manually inoculated with leaf extracts of *C.quinoa* infected with SLRV-D, and grown at three different temperatures, 18°, 25° and 28°C in growth cabinets illuminated at light intensity of 7,000 lux for 16 h/day.

As only two temperatures could be tested at a time, the experiment was done with 18°C as control. Four plants were tested at each temperature and all the plants were indexed for virus soon after flowering in order to confirm the presence of SLRV at the time of seed set.

Seeds were then collected from individual plants,and germinated. Seedlings were indexed for virus on *C. quinoa*, and the results are given in **Tables 39** and **40**.

Table 39 Effect of temperature on the transmission of SLRV-D through seeds

Host species	18°C	25°C
C.quinoa	+100 (60)	74 (80)
N.rustica	60 (57)	69 (60)

+Percentage infection

Figures in parenthesis are total numbers of seedlings tested.

Table 40 Effect of temperature on the transmission of SLRV-D through seeds

Host species	18°C	28°C
C.quinoa	+100 (60)	28 (80)
N.rustica	68 (57)	40 (60)

+Percentage infection

Figures in parenthesis are total numbers of seedlings tested.

The results showed that higher post-inoculation temperatures (25° or 28°C) reduced the seed transmission of SLRV in *C. quinoa* and in *N. rustica* (28°C). The differences in transmission in *C. quinoa* ,

were significant ($P < 0.05$, $\chi^2 = 34.5$) at 25° and 28°C.

The reduced percentage seed transmission at higher temperatures may probably be due to a low levels of SLRV in the plants. This was evidenced by the DEP in ELISA detected two weeks after inoculation, which was between 1/600-1/1,200 compared to that of those plants kept at 18°C (1/9,600+).

In further experiments post-inoculation temperatures of 18° and 33°C were compared. ***C. quinoa*** and ***N. rustica*** plants were inoculated with the same inoculum prepared from ***C. quinoa*** leaves with SLRV-D. All inoculated plants were kept at 18°C for 4 days to allow the virus to establish in plants. Some plants of each species were then transferred to a growth cabinet at 33°C, until seed set. The plants at high temperature were indexed for virus on ***C. quinoa*** at weekly intervals. To avoid temperature stress during seed set,these plants were transferred again to 18°C about three weeks after flowering.

Seeds from all plants were collected separately and seed transmissibility assessed by indexing 40 seedlings from each treatment individually on ***C.quinoa***.

Table 41 Effect of post-inoculation temperature on seed transmission of SLRV-D

	% virus transmission	
Host	18°C	33°C
C. quinoa	100	0
N.rustica	45	23

The results (**Table 41**) showed that seed transmission of SLRV was affected by post-inoculation temperature and the virus was eliminated from the seeds of *C. quinoa* set at 33°C . The virus could not be detected in those plants kept at 33°C after the first week, when assayed on *C. quinoa*. In *N. rustica* virus transmission at 33°C was about 50% that of seeds produced at 18°and virus could not be detected in mother plants after the first week at 33°C. It seemed, therefore, that SLRV was eliminated from mother plants kept at 33°C, presumably because at this temperature virus synthesis was inhibited, while the degradation of existing virus continued (Walkey, 1985).

6.4 Effect of age of *C. quinoa* at infection on seed transmission of SLRV-D

Four *C.quinoa* plants grown at 20 - 24°C were selected at random, seven, nine and eleven weeks after germination, and inoculated with SLRV-D. Another four plants were inoculated after flowering (**Fig. 40**). All plants were inoculated with inocula at 1:1 / w:v and grown at 18°C until seed set, then transferred to glasshouse at 20 - 24°C for drying. Seeds were collected separately from plants of each treatment and pooled. After germination seedlings from each treatment were assessed for SLRV by ELISA using 150-200 seedlings tested in groups of five. The results are given in **Table 42.**

Fig. 40 *C. quinoa* with flower heads at the time of inoculation.

Table 42 Effect of age at infection on seed transmission of SLRV-D in *C. quinoa*

Age at infection (weeks)	*No.of groups giving positive reaction
07	19/20
09	25/25
11	20/20
14 (after flowering)	30/30

* no.of positive groups/no.of groups tested.

The results showed that there was little effect of age at infection on seed transmission of SLRV. Those plants inoculated after flowering showed mild chlorotic mottle symptoms (systemic) as in younger *C. quinoa* plants kept at 18°C. It was also evident that SLRV was able to infect the seed when inoculated to mature plants even after flowering when kept at a temperature favourable for virus establishment.

6.5 Pollen transmission of SLRV

Pollen collected from SLRV-D infected parsnip and other experimental hosts was macerated separately in few drops of inoculation buffer and indexed for SLRV on *C. quinoa* (Chapter 2.6). The results are shown in **Table 43.**

Table 43 Presence of SLRV-D in pollen of infected plants

Host species	Symptoms	Infection in *C. quinoa*
C.quinoa	+	+
S.oleracea	+/-	+
Coriandrum sativum	-	+
P.sativa 'White Gem'	+/-	+
N.glutinosa	-	+
N.rustica	-	+
N.debneyi	-	+
N.clevelandii	+/-	+

+/- symptoms sometimes present

The results showed that the pollen of infected hosts carried the virus whether the infection resulted in severe symptoms (+) or was symptomless (-).

To determine whether SLRV could enter the seed via infected pollen, naturally-infected parsnip mother plants were kept adjacent to healthy parsnip plants to allow cross pollination. Pollen from infected plants was also dusted onto flowers of healthy plants to allow pollination. Infected and healthy coriander plants were allowed to cross pollinate in a similar manner.

However, when seeds (about 75) collected from cross pollinated healthy parsnip and coriander plants were back tested on *C. quinoa* seedlings, no infection was detected. As these species are usually self pollinated, (Walkey, 1985) and the incidence of cross pollination was uncertain in the method used, a larger number of seed

samples may have been required to detect virus if low levels of transmission occurred.

In a further experiment to determine whether seed could be infected from pollen, cucumber which is systemic host for SLRV and has unisexual flowers, was used.

About 10-12 cucumber cv Marketer seedlings were inoculated with SLRV-R and grown in a glasshouse at 20 - 24°C. Uninoculated plants were also grown in the same glasshouse. Shortly after flowering all female flowers, both diseased and healthy, were covered with pieces of muslin in order to prevent any unwanted pollinations. The flowers were hand pollinated using a paint brush to collect pollen from male flowers and deposited them carefully on the female flowers, at least twice, on two successive days. Different brushes were used in different crossings and the pollinated flowers were immediately covered with a polythene bag to prevent subsequent contamination. Plants were left to set seed, and the seeds were separately harvested from ripened fruits.

Seeds were indexed on ***C.quinoa*** either directly in groups of ten or after germination as seedlings in groups of two. The results are shown in **Table 44.**

Table 44 Transmission of SLRV-R in cucumber seeds produced by different crosses.

Crossing type ♂ x ♀	% Seed transmission
H x H	0
H x D	40-60
D x D	90-100
D x H	40

H - Healthy
D - Diseased

The results showed that the virus was able to enter the embryo from infected pollen (40%). Similar levels of transmission (40-60%) were found when healthy pollen was used to fertilise infected ovules. When leaves from healthy plants cross pollinated with diseased pollen were tested for SLRV by ELISA, there was no indication that the pollinated plant had become infected.

6.6 Effect of SLRV infection on pollen production

In this experiment to investigate the effect of SLRV on pollen production ***N. clevelandii*** was selected as it has fairly big anthers which could be handled easily.

Five ***N. clevelandii*** seedlings (four leaf stage) were inoculated with SLRV-R infected ***C.quinoa*** leaf sap and kept in a glasshouse at 20 - 24°C to set seed.

Fig. 41 Cucumber seed resulting from different crossing types
(a) ♂H x ♀H (b) ♂D x ♀H (c) ♂D x ♀D

Another set of five plants of similar age were left uninoculated in the same glasshouse, care being taken to avoid contamination with pollen grains from infected plants. When plants had flowered, two mature anthers (before dehiscence) from two different flowers were collected from each plant.

Ten anthers collected from each set of plants were macerated in 0.1 ml water and the suspension diluted up to two ml with 50% glycerol (Chapter 2.6). The number of pollen grains were counted in one ml sub samples, in 16 randomly chosen wells of counting chamber (Chpter 2.6). The results are shown in **Table 45.**

Table 45 Pollen production in ***N.clevelandii*** infected with SLRV-R

	No. of pollen grains	
	infected	healthy
Total No. of pollen grains in 16 µl	23	81
Estimated No.of pollen grains in 2 ml (10 anthers)	2,875	10,125

The experiment was repeated with a similar set of plants and pollen was counted from eight anthers of each set, made up to one ml final suspension. Pollen grains in 24 wells were counted and the estimated numbers in infected and healthy preparations were 16,875 and 28,458, respectively.

These results showed SLRV-R infection had affected pollen production in ***N. clevelandii***. According to the results of the first experiment, there was a 71.6% decrease in pollen production in SLRV-infected plants, although in the second experiment it was only 40.7 % under the conditions tested (these experiments were not done in a controlled environment).

6.7 Effect of SLRV-R on *in vitro* germination of infected pollen

Three experimental hosts - cucumber, ***N. clevelandii*** and ***N. tabacum*** cv. Xanthi were used in this experiment. Two plants of each species were manually inoculated with SLRV-R infected leaf extracts of ***C.quinoa***. These plants together with two uninoculated control plants of each species, of similar age, were grown in a glasshouse at 20 - 24°C. Anthers in eight individual flowers from the two plants (four from each plant) were collected and a pollen suspension made in two ml of 30% sucrose solution cotaining 100 mg of boric acid (Chapter 2.6). Pollen was allowed to germinate overnight in this medium and the number of germinated pollen grains in one ml samples was counted using a counting chamber. The results are shown in **Table 46** and **Fig. 42a** and **42b**.

Fig. 42a *In vitro* germinated pollen from healthy *N. clevelandii* , 3h after incubation.

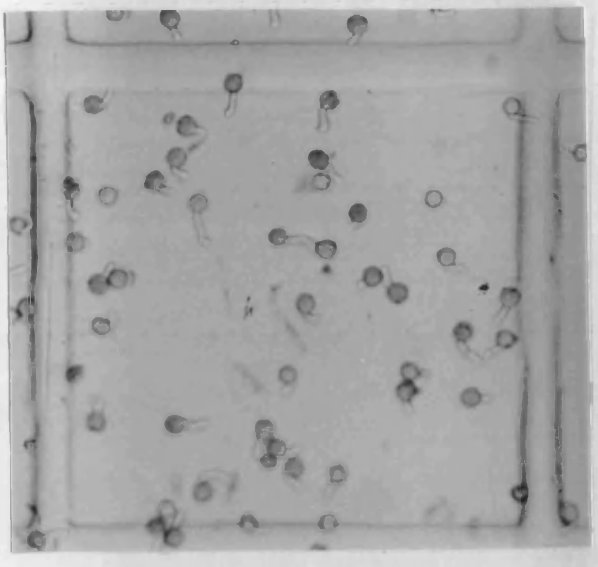


Fig. 42b *In vitro* germinated pollen from SLRV-R infected *N. clevelandii* 3h after incubation.

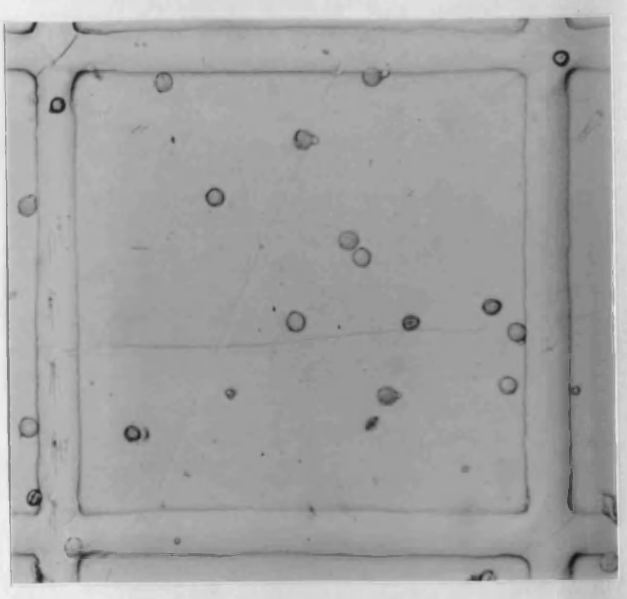


Table 46 Percentage germination of pollen (*in vitro*) from healthy and SLRV-R infected hosts

Host	[a] Mean % germination	
	Infected	Healthy
N.clevelandii	8.7	18.3
N.tabacum 'Xanthi'	18.0	20.9
C.sativus 'Marketer'	14.5	36.6

(a) in six randomly chosen wells

The results indicated that pollen germination was reduced in ***N. clevelandii*** and in cucumber infected by SLRV but not in ***N. tabacum 'Xanthi'***, Furthermore, in ***N.clevelandii*** shorter pollen tubes and low rate of germination were noted (**Fig. 42b**). However, there was no apparent difference in the tube length of cucumber pollen infected with SLRV compared with healthy pollen. These results suggested, therefore, that the effect of SLRV on pollen varied with host species infected. It also suggested that in ***N. clevelandii*** infected pollen would probably be less competitive than healthy pollen.

CHAPTER 7 Effects of SLRV infection on yield.

Test hosts used in these experiments were beetroot, and spinach, both of the family Chenopodiaceae, and coriander and parsnip, of the family Umbelliferae. Root yield was determined for beetroot and parsnip, whereas seed yield was determined in coriander and spinach.

7.1 Effect of SLRV-R on the root yield of beetroot

Beetroot seedlings at the four leaf stage were inoculated with buffered extracts of *C. quinoa* leaves infected with SLRV-R (1:1 / w:v). These seedlings, which were in 10 cm pots, were then randomised in a glasshouse at 20-24°C. A uniform set of similarly-aged plants inoculated with buffer only was also randomized with the inoculated plants in the same glasshouse. Care was taken to keep all other factors (watering, fertilisation etc.) as homogeneous as possible. Five weeks after inoculation, all plants were re-potted in 28cm pots. Although SLRV-R readily produced local and systemic symptoms in beetroot, a few plants were infected symptomlessly. All 20 inoculated beetroot plants, therefore, were back tested on *C. quinoa* one week before harvesting, to confirm plants were still infected with the virus at the time of harvesting.

Roots were lifted 14 weeks after inoculation, and after rinsing off soil particles, were thoroughly blot-dried before weighing. The leaves (removed from the base) and roots, were weighed separately and the fresh weights are shown in **Table 48.**

The differences in mean fresh weight were highly significant ($P < 0.01$) and it was evident that SLRV-R infection had a marked effect on the root yield of beetroot. The loss in crop yield in this experiment was about 55%. The reduced size of roots from infected plants was clearly seen (**Fig. 43**).

Table 48 Effect of SLRV-R on the root yield of beetroot

Weight (g)	
SLRV-R infected	uninoculated
16.52	58.26
14.57	74.74
35.18	56.22
38.69	56.69
24.64	71.36
30.88	45.57
21.39	73.06
17.29	40.53
18.96	39.79
13.07	62.69
34.64	44.76
32.17	34.45
11.12	39.94
04.25	54.26
15.46	44.27
32.33	32.04
31.17	42.25
10.29	34.38
17.92	29.04
	60.61
Total 420.54 (19)	997.41 (20)
Mean 22.13g	49.87g

Fig. 43 Beetroots harvested 14 weeks after inoculation with SLRV-D. Uninoculated healthy plants on right.

7.2 Effect of SLRV-D on the seed yield of *Spinacea oleracea*

In this experiment, seed yield of *Spinacea oleracea* inoculated with an 'intermediate' isolate(SLRV-D) was compared with that of healthy plants.

A set of eight uniform spinach plants at the four leaf stage were inoculated with leaf extracts of *C. quinoa* infected with SLRV-D. These plants were randomized together with another set of eight plants which were also of the same age but left uninoculated. Both lots of plants were kept at 20-24°C in a glasshouse to set seed.

Although SLRV-D in spinach produced a slight systemic chlorotic mottle on leaves, at lower post-inoculation temperatures plants were often symptomless. All inoculated plants, therefore, were back tested on *C. quinoa*, two weeks after inoculation and again at flowering.

Seeds were harvested from both sets of plants and the seed weights are given in **Table 49.**

Table 49 Effect of SLRV-D infection on seed yield of spinach.

	Total weight (g)	Mean yield per plant (g)
SLRV-D	23.5(a)	4.70
Healthy	68.7	8.58

(a) weight of seeds produced by five infected plants.

All eight plants became infected with SLRV-D but three plants were killed shortly after flowering. Therefore, the results given in **Table 49** was the total fresh seed weight of five infected plants. The results were significant ($P<0.05$) and it showed there was about 66% loss in seed yield by SLRV infection in spinach.

7.3 Effect of SLRV-D infection on the seed yield of *Coriandrum sativum*

Coriandrum sativum mechanically inoculated with SLRV-D was used in this experiment. Both inoculated and healthy controls were grown for seed in a glasshouse at 20-24°C taking care to keep all other factors (watering, fertilization etc.) as homogeneous as possible. Seeds were harvested from each sets of plants and the results are shown in **Table 50.**

Table 50 Effect of SLRV-D on the seed yield of manually inoculated coriander

	No.of seeds	
	SLRV-D infected	Healthy
	164	269
	163	552
	216	379
	140	267
	125	292
	136	254
Mean / plant	158.8	335.5

The results were significant ($P < 0.05$) and the number of seeds produced in SLRV-infected plants was reduced by about 53% when compared to that in healthy plants.

A comparison of the effects of a severe (I) and an 'intermediate' (D) isolate of SLRV, on the seed yield of coriander

An attempt was made to determine the effect of different isolates of SLRV on the seed yield of coriander. The experiment was performed as described above, but using natural seed-borne infection rather than mechanically-inoculated plants. The seeds were collected from coriander mother plants infected with SLRV-I or D by mechanical inoculation. Seedlings of the F1 progeny were indexed for virus on *C. quinoa* and seven plants infected with each isolate were then grown for seeds in a glasshouse at 20 – 24°C. Seeds were counted separately for each plant and the results are shown in **Table 51.**

Table 51 Effect of two SLRV isolates on the seed yield of coriander infected through seeds

	Number of seeds		
	SLRV-D	SLRV-I	Healthy
	21	47	29
	11	23	114
	06	41	105
	12	51	136
	48	63	43
	15	38	156
	58	24	255
Mean/plant	24.4	41.0	119.7

The results showed that both seed lots infected with SLRV-I and SLRV-D were significantly ($P < 0.05$) reduced in number compared to healthy seeds. However, there was no significant difference between the two isolates with respect to number of seeds produced although those seeds infected with SLRV-I were smaller, malformed and underdeveloped (**Fig.44**) compared to seed from plants infected with SLRV-D, or seed from healthy plants.

7.4 Effect of SLRV-D and I on the root yield in parsnip.

Parsnips (cv. White Gem) were raised from naturally-infected seeds collected from mother plants infected with SLRV-D or I. Attempts to infect parsnips mechanically with leaf extracts of *C. quinoa* or parsnips infected with SLRV were unsuccessful at the time this experiment was done.

Seeds collected from infected and healthy mother plants were sown, to give about 5 - 6 seeds / pot (10 cm pots); On germination extra seedlings were removed to leave one good plant/pot. Seedlings which developed from infected seed lots were back tested for virus on *C.quinoa*. When infection was confirmed, twenty pots from each seed lot were fully randomised in a glasshouse at 20 - 24°C.

Eight weeks after sowing, plants were re-potted into 28 cm pots, and grown for ten months. During

Fig. 44 Effect of SLRV-I on coriander. (a) healthy, (b) infected

this period all the pots were treated equally with respect to watering, fertilization etc. After ten months the roots were harvested, soil particles rinsed off, and the roots blot-dried before weighing. The results are given in **Table 52.**

Infected parsnip plants were indexed at monthly intervals on *C. quinoa* up to two weeks before harvest. In the last two tests SLRV could not be detected in leaves of four plants which were originally known to be infected. The results given in **Table 52** therefore, are the figures of 16 plants which were infected at the time of harvesting.

Table 52 Effect of SLRV-D infection on parsnip root yield

	Weight of root (g)	
	SLRV-D	Healthy
	73.4	194.4
	128.4	196.5
	108.6	124.4
	83.9	146.3
	105.6	170.7
	133.7	146.1
	137.2	153.9
	127.6	208.0
	157.7	167.3
	198.6	180.1
	124.1	198.6
	180.5	131.2
	193.7	188.7
	173.5	206.5
	110.1	141.7
	85.9	140.4
		172.0
		100.1
		172.0
		205.8
Mean wt/root	132.6 ± 39.0	167.2 ± 30.8

The difference in yield, about 20%, was not significant ($P > 0.05$) and the infected roots appeared normal when compared to healthy roots.

In the next experiment, the severe isolate, SLRV-I was assessed for its effect on root yield of parsnip as described for isolate D; the results are given in **Table 53.**

Table 53 Effect of SLRV-I infection on rot yield of parsnip

	Weight of tap root (g)	
	SLRV-I infected	Healthy
	101.34	122.20
	121.76	100.37
	111.28	100.97
	114.50	123.73
	152.25	97.59
	174.31	150.79
	104.58	121.06
	70.91	94.57
	97.92	116.24
	80.38	114.32
	131.62	114.35
	197.66	93.90
	155.93	140.25
	138.54	121.13
	121.22	133.73
		111.84
		117.85
		135.11
		140.38
		149.73
Mean wt./root	124.95 ± 34.2	120.01 ± 17.5

Although 20 plants infected through seeds were tested initially, the virus could not be detected in the leaves of five plants when indexed to *C. quinoa* in the last two tests before harvesting. Therefore, the figures given in **Table 53** are of 15 infected plants.

These results showed that there was no significant effect of the infection by isolate I on the root yield of parsnip. It is evident from these two experiments, therefore, that SLRV infection did not affect the root yield of parsnips under the conditions tested.

The fact that SLRV could not be detected in some plants during the later stages of growing may have been due to the high temperature in the glasshouse. In the first experiment, the roots were harvested in September 1988 and that of the second in August 1989, when the glasshouse temperatures in these summer periods were often above 30°C. It is possible, therefore, that virus concentrations had decreased from some plants, or virus elimination had occurred. Differential effects of temperature on virus concentration in individual roots may be reason for the wide variation in the root weight of infected plants especially in experiment two, with SLRV-I. In future experiments it would be interesting to compare root yields under sub-optimal conditions, in the field, and to extend the comparisons over two years.

CHAPTER 8 *In vitro* techniques for eradication of SLRV.

8.1 Thermotherapy

High temperature treatments combined with tissue culture techniques have been widely used for the production of virus-free plants (Stone,1968 ; Nyland and Goheen,1969 ; Walkey ; 1976 ; Mantell ***et al.***,1980). Heat therapy normally involves the infected parent plant or organ of the plant being grown in temperature controlled cabinets (30-40°C) for various periods (Walkey, 1985). When the plants are grown in high temperature, the leaves may be exposed to slightly higher temperature than the roots so that the virus is inactivated /eliminated from the leaves or shoots but not from the roots (Pennazio ***et al.***, 1976). In these circumstances it is essential to remove potentially virus-free shoots/organs from the heat treated plants and to grow them as an explants ***in vitro*** to produce healthy plants (Walkey, 1985).

Seed-borne infection has been identified as one of the most important sources of infection from which many nepoviruses including SLRV spreads into a crop (Murant, 1983). With SLRV in parsnips, the main source of infection seemed to be the crop itself and was due to the introduction of infected seeds (Cooper, 1981; Hicks ***et al.***, 1986). In the present study SLRV was readily isolated from two sources of commercial seed not only of

'White Gem' but also from cv. Tender and True (see Chapter 3.1). Both cvs. were marketed by same firm (Suttons Seeds Ltd.) and were probably grown on the same field site.

If seeds of cultivar like 'White Gem' are widely infected with SLRV then a simple procedure for elimination of virus from infected seeds or seedlings would be valuable. This would enable new stocks of seed from virus-free mother plants to be produced to replace existing contaminated supplies.

Several attempts were made to inactivate SLRV in seeds by subjecting them to high temperature treatment before germination ***in vivo***. Seeds of ***C. quinoa*** and ***Pastinaca sativa*** 'White Gem' plants infected with SLRV-D, in which the virus transmission were 100% and 80-90% respectively, were used for these experiments with untreated infected seeds as controls. Seeds were treated either with hot water or hot air, slightly above the TIP of the virus for 20 min, or slightly below the TIP for longer periods. After treatment, seeds were germinated ***in vivo*** and indexed for virus on ***C. quinoa*** . None of these heat treatments, however, was successful in the inactivation of virus (**Table 54**).

Table 54 Effect of heat treatments on the inactivation of SLRV-D in the seeds of *C. quinoa* and parsnip

Seed	Treatment	%germination	Infectivity(c)	
			Treated	untreated
C.quinoa	60°C (20 min)[a]	0 (100)	-	-
C.quinoa	45°C (16h)[a]	100 (100)	40/40	40/40
C.quinoa	45°C (24h)[b]	100 (98)	20/20	19/20
Parsnip	45°C (48h)[b]	56 (55)	32/40	31/40

a Hot water
b Hot air
(c) No. of infected seeds / No. of seeds assayed

Figures in parenthesis are data for untreated seeds.

The results showed the heat treatment at 60°C killed the seeds of *C. quinoa* . A longer incubation at a lower temperature (45°C) had no effect on the viability of *C. quinoa* but this temperature was too low or the incubation too short to eliminate virus from the seed.

The next experiment was to determine whether SLRV could be inactivated or eradicated from germinating *C. quinoa* and *P. sativa* seeds heated *in vitro* , following a technique used by Cooper and Walkey, (1978) to eradicate cherry leaf roll virus in infected *N.clevelandii* seeds.

Seeds were surface sterilized in 5% sodium hypochlorite solution and transferred to culture media aseptically (Chapter 2.16). Cultures (at least 10 from

each species) were kept in growth cabinets at 33 ± 1°C and the controls at 20 ± 1°C, illuminated 16h / day with six fluoresent tubes 8 Watts. Although the seeds of ***N.rustica*** and ***C.quinoa*** started to germinate within 24-48h at both temperatures, the rate of germination of parsnip seeds was found to be very slow at 33°C. Both sets of parsnip culture tubes, therefore, were initially kept at 20°C, and half of the tubes transferred to 33°C immediately after germination (normally 5 - 10 days). The duration of the heat treatment (two weeks) was counted from the day on which the tubes were transferred to the high temperature.

All cultures were tested for virus two weeks after germination by indexing to ***C. quinoa*** and the results are given in **Table 55.**

Table 55 Transmission of SLRV isolates in the seed cultures of ***N.rustica, C.quinoa*** and ***P.sativa*** grown at 20°C and 33°C.

Host/virus	Infectivity (a) 20±1°C	33±1°C
N. rustica /SLRV-R	20/28	0/30
C. quinoa / SLRV-D	40/40	0/40
P. sativa / SLRV-D	7/10	0/10

(a) No.infected/ No. assayed

The results showed that SLRV had been inactivated by 100% in all three cultures kept at 33 ± 1°C for about two weeks in all host/isolate combinations. This

length of time was the same as that needed to inactivate virus from mechanically inoculated plants kept at the same temperature (Chapters 3.8 and 6.3).

In the next experiment, the minimum time required for thermal inactivation of virus in seed cultures was determined. Fifty seed cultures of each species were raised at the same temperature and ten cultures were assayed by indexing on *C. quinoa* , at four days intervals, over a period of 20 days. The results are shown in **Table 56.**

Table 56 Minimum time required for 100% inactivation of of SLRV in seed cultures raised at 33±1°C.

* Infectivity

Days in culture	*N.rustica* /R	*C.quinoa* /D	*P.sativa* /D
4	7/10	10/10	7/10
8	8/10	10/10	4/10
12	3/10	4/10	0/10
16	0/10	0/10	0/10
20	0/10	0/10	0/10

* No. infected / No. tested

The results showed that SLRV had apparently been eliminated between 12-16 days in cultures of *C. quinoa* and *N. rustica* and between 8-12 days in parsnip. However,it has been reported (Walkey, 1985) that following thermotherapy with many host / virus combinations, infection may reappear later. For example,

cucumber mosaic virus and alfalfa mosaic virus were recovered from cultured tissues of *N. rustica* , following heat treatment (Walkey, 1976).

A further experiment therefore, was done to determine the length of heat treatment required for a complete eradication of SLRV from the same three host plants used in the previous experiment.

Seed cultures were grown at 33±1°C, and ten cultures from each species were transferred to a growth cabinet at 20±1°C at four days intervals. Transferred cultures were kept at this temperature for 10-52 days before assaying for virus. The results are given in **Tables 57, 58** and **59.**

Table 57 Presence of SLRV-R in heat-treated *N.rustica* seed cultures after transfer to 20°C.

Days in culture 33°C + 20°C (*N.rustica*)	Heat treated	Control[b]
4 + 10	[a] 8/10	[a] 8/10
8 + 10	8/10	8/10
12 + 12	3/10	7/10
16 + 12	0/10	7/10
20 + 16	0/10	8/10
28 + 32	0/10	6/10
36 + 52	0/10	7/10

[a] Assayed by ELISA, No. infected/ No. tested
[b] Infected seed germinated continuously at 20°C

Table 58 Presence of SLRV in heat-treated *C. quinoa* seed cultures after transfer to 20°C.

Days in culture 33°C + 20°C (*C. quinoa*)	Heat treated	Control[b]
4 + 10	[a]10 / 10	[b]10 / 10
8 + 10	10 / 10	10 / 10
12 + 10	8 / 10	9 / 10
16 + 14	0 / 10	10 / 10
20 + 22	0 / 10	10 / 10
24 + 32	0 / 10	10 / 10

[a] Assayed by ELISA, No. infected/No. tested
[b] Infected seeds germinated continuously at 20°C

Table 59 Presence of SLRV in heat-treated *P. sativa* seed cultures after transfer to 20°C.

Days in culture 33°C+20°C (*P.sativa*)	Heat treateted	Control[b]
4 + 10	[a]7 / 10	[a]7 / 10
8 + 12	5 / 10	8 / 10
12 + 12	0 / 10	8 / 10
16 + 16	0 / 10	7 / 10
20 + 22	0 / 10	8 / 10
24 + 20	0 / 10	7 / 10

[a] Assayed by ELISA, No. infected/ No. tested
[b] Infected seed germinated continuously at 20°C

These results showed that in *C. quinoa* and *N. rustica* SLRV was apparently eliminated from tissues cultured from seed between 12 - 16 days at 33°C, although in parsnip inactivation took place between after 8 - 12 days, possibly because virus concentrations were lower.

In later experiments, however, after heat treated parsnip cultures had been potted into compost and kept in a glasshouse at 20-24°C for two - three months. SLRV was detected by ELISA in the leaves of four out of ten parsnip plants which had previously indexed negative. In further experiments, duration of heat treatment was extended to 36 days but still SLRV was detected by ELISA in about 30 - 40 % parsnips after transfer to 20°C. Therefore, it seems an extended heat treatment may be required to give complete elimination of SLRV from all infected parsnip seeds.

8.2 Chemotherapy of SLRV-R in *in vitro* seed cultures

Although several antiviral chemicals have potential in inhibiting virus multiplication,(Dawson,1984) only ribavirin (1-β-D-ribofuranosyl-1,2,A-triazol-3 carboxamide) was used in the present work.

The same medium was used as in thermotherapy experiments, but filter-sterilized ribavirin was added to the medium after autoclaving (Chapter 2.15). Infected seeds of ***N. rustica*** and ***P. sativa*** were grown in the medium at 20 ± 1°C. Infected seed of each host grown in ribavirin-free media was used as a control. Seedlings were grown for three to four weeks and then assayed for virus by ELISA. The results are given in **Table 60.**

Table 60 The effect of ribavirin on incidence of SLRV-R in ***N. rustica*** seeds cultures grown ***in vitro***

Ribavirin concentration (mg/l)	% Germination	% Infection	
		Expt.1	Expt.2 (a)
10	92	64.7	50
30	96	76.9	75
50	97	72.7	80
0	96	75.7	70

(a) Assayed by symptom production in ***C. quinoa***

The results showed that there was a 14.5% inhibition of SLRV in experiment one and 28.6% in experiment two, in the media containing 10 mg/l ribavirin. However, higher concentrations of ribavirin had no inhibitory effect on SLRV while the plants had a slightly lower growth rate. In an initial trial, it was observed that ***N.rustica*** seeds could grow in the medium with 75, and 100 mg/l ribavirin although growth rates were poor and no virus inhibition was found at those higher concentrations.

In contrast, parsnip seeds did not germinate in the media containing 30 or 50 mg / l ribavirin and there was only about 15 - 20% germination in 10 mg/l and seedlings had a poor growth rate. The experiment was repeated, therefore, using lower ribavirin concentrations of 0.1 - 1.0 mg / l. Seedlings (about 15 - 20) were tested for virus after 4 weeks at 20 ± 1°C and

the results are given in **Table 61.**

Table 61 The effect of ribavirin on incidence of SLRV-D in *P. sativa* seed cultures grown *in vitro*

Ribavirin concentration (mg/l)	% germination	% parsnip seedlings infected (a)
0.1	57	58
0.5	44	94
1.0	45	90
0	56	82

(a) Assayed by ELISA

These results showed there was about 29% fewer seedlings infected with SLRV-D in 0.1 mg/l ribavirin but no effect of the other two (0.5 and 1.0 mg/l) concentrations. At all concentrations the seeds germinated normally and after four weeks in the culture medium there was no evidence of reduced vigour. As with heat treated seed cultures, apparently virus-free ribavirin treated plants should be monitored continuously for any reappearence of virus, once taken out of the medium containing ribavirin.

CHAPTER 9 Characterisation of Parsnip yellow fleck virus (PYFV)

9.1 Isolation and identification of PYFV-E

Only one parsnip plant out of 17 growing in the field, tested for virus (Chapter 3.1) was infected with PYFV. the presence of this virus was suggested by the production of small necrotic local lesions on *C. quinoa* (Murant, 1974) and the absence of the characteristic systemic chlorotic mottle / necrosis produced by SLRV. Gel double-diffusion tests confirmed the presence of PYFV. This isolate was characterised further with respect to its biological, physical and chemical properties.

Severe symptoms associated with PYFV infection were observed in the field (Chapter 3, **Table 2**) but the plant died two months after being transplanted into 28 cm. pots and kept in glasshouse at 20-24°C, so further observations could not be made.

9.2 Host range PYFV-E

Leaf extracts of *C. quinoa* infected with PYFV-E were manually inoculated (in 0.05 M phosphate buffer, pH 7.6)to a range of test plants and grown in a growth cabinet at 18°C illuminated at 7,500 lux for 16/h day. Those plants which did not show any symptoms four weeks after inoculation, were back tested on *C. quinoa* for

symptomless infection in both inoculated and uninoculated leaves. The results are shown in **Table 62.**

Table 62 Host range of PYFV-E

Plant species	Symptoms
Chenopodiaceae	
C.quinoa Willd	NL,SF,D
C.amaranticolor Coste and Reyne	NL,SF,D
C.foetidum Schrad	L,SO
S.oleracea	CL,SM,D
Umbelliferae	
Daucus carota L.	-
Apium graveolens var. dulce	L,SF
Coriandrum sativum	L,SN,K
Pastinaca sativa cv. White Gem	L,SM
Anthriscus cerefolium	L,SN,K
Anethum graveolens	-
Solanaceae	
N.tabacum L.'White Burley'	CL,SO
N.tabacum 'Xanthi'	CL,SO
N.clevelandii Gray.	CL,SM
N.glutinosa L.	SO
Datura stramonium L.	-
Petunia multiflora	-
Cucurbitaceae	
Cucumis sativus L. 'Marketer'	L
Amaranthaceae	
Gomphrena globosa L.	NL
Leguminoseae	
Phaseolus vulgaris L. 'Prince'	-
Vicia faba L.	-
Pisum sativum 'Meteor'	-
Phaseolus multiflourus	L
Cruciferae	
Brassica campestris sub sp. 'Chinensis'	-
Lactuca sativa	-

L, Local infection
SO, symptomless systemic infection
SM, Systemic mottle
SN, Systemic necrosis
SF, Systemic flecking
NL, Necrotic local lesions
D, Distortion of leaves
K, Plant death
-, No infection
CL,Chlorotic local lesions

The symptoms of PYFV-E varied from local chlorotic spots to chlorotic mottle or necrosis, leaf distortion and even death of plants. Symptom expression of PYFV-E also varied with post-inoculation temperature. Symptoms were hardly visible at 25°C (in growth cabinets) and in summer in the glasshouse the virus was difficult to propagate in any host. Temperatures of about 18 C were ideal for symptom expression of PYFV.

More detailed symptom descriptions on selected test plants are given below:

C.quinoa - With a concentrated leaf inoculum from ***C.quinoa*** about 25-30 necrotic lesions of 1-2mm were produced on inoculated leaves, 4-5 days after inoculation. This was followed by systemic chlorotic flecks, and especially in young seedlings, leaf distortion. Plants were stunted (**Fig. 45a**).

N.clevelandii - Local chlorotic spots surrounded by necrotic rings were produced within 8-10 days of inoculation. A systemic mottle was observed in about 15-20 days, and later dark green blotches developed. No apparent stunting was observed when compared to healthy ***N.clevelandii*** (**Fig.45b**) plants.

Spinacea oleracea - PYFV-E produced local chlorotic spots followed by a severe systemic mottle and infected plants were stunted (**Fig. 45c**).

Coriandrum sativum - This was the most susceptible of all the plants tested. Local necrotic spots were produced in 6-8 days and the newly emerged leaves were blackened and shrivelled. The plants were killed by infection in about two weeks (**Fig. 45d**).

Apium graveolens - PYFV-E produced local chlorotic spots followed by a severe systemic fleck. Leaves distorted and reduced in size compared to healthy hosts (**Fig. 45e**).

9.3 Physical properties of PYFV-E

The DEP,TIP and LIV of PVFV-E were determined using infected *N. clevelandii* as virus source and *N. tabacum* cv. White Burley as assay host plants. In *N. tabacum* (**Fig.45f**) the virus produced about 30-40 clear chlorotic lesions (depending on the strength of inoculum) of 4-6 mm in diameter on inoculated leaves. This host was originally considered as a local lesion host only because the crude sap from systemic leaves neither infected *C. quinoa* nor reacted with an antiserum to PYFV in gel diffusion tests. However, PYFV was detected later when the systemic leaves were partially purified (Chapter 2.10) and tested in gel-diffusion.

For the determination of physical properties of PYFV-E *N.tabacum* cv.White Burley plants were used as a local lesion assay host and the experiments were

Fig. 45a Local necrotic lesions induced by PYFV-E in *C.quinoa* .

Fig. 45b *N. clevelandii* infected with PYFV-E showing systemic chlorotic mottle, three weeks after inoculation.

Fig. 45c *Spinacea oleracea* (spinach) infected with PYFV-E

Fig. 45d Coriander infected with PYFV-E showing die-back symptoms. (a) three weeks after inoculation (b) six days after inoculation (plant blackened with shrivelled leaves).

a b

Fig. 45e Leaf distortion and chlorotic fleck induced by PYFV-E in celery, healthy plant on right

Fig. 45f Local chlorotic lesions induced by PYFV-E in *N. tabacum* cv. White Burley

done as described in (Chapter 2.9). The results are given in **Table 63** and **Fig 46**.

Table 63 Physical properties of PYFV-E

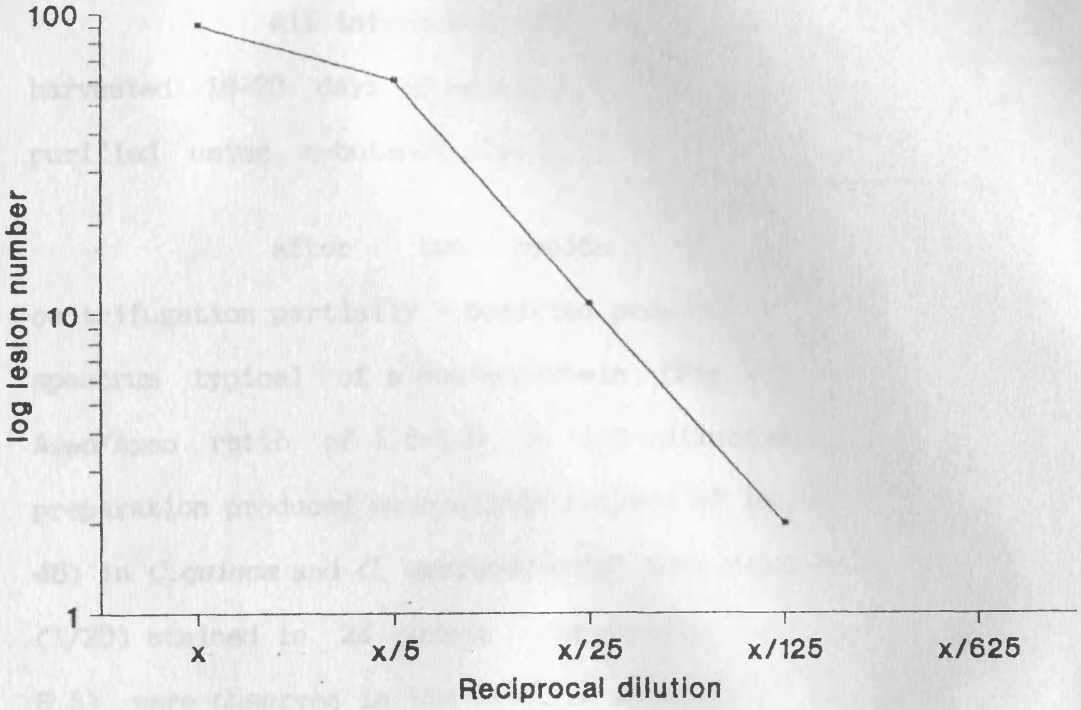
	TIP (°C)	DEP	LIV (Days)
Expt.1	55-60	10^{-2} - 10^{-3}	1-2
Expt.2	54-57	1/125 - 1/625	2-4

The results indicated that PYFV was a moderately unstable virus that reached relatively low concentrations in sap extracts. These results were generally similar to the published data of Murant and Goold (1968) for PYFV although they recorded a slightly higher DEP for their isolate. However, a critical comparison of these properties is difficult because many factors (host species, environment etc.), other than intrinsic differences between the isolates, can affect the results.

9.4 Purification of PYFV-E

Attempts were made to purify PYFV-E to study particle properties and also to make an antiserum. Leaves of *N. tabacum* 'White Burley' plants were inoculated with PYFV-E. When local lesions formed 5-6 days after inoculation, individual lesions were used to establish a single lesion isolate of PYFV-E as described in Chapter

Fig. 46 Dilution curve of parsnip yellow fleck virus



X = 1:0·5/W:V

2.4. After two successive single lesion subcultures in tobacco, the virus was bulked in ***N. clevelandii***.

All infected leaves of ***N. clevelandii*** were harvested 18-20 days after inoculation and PYFV-E was purified using n-butanol clarification (Chapter 2.10).

After two cycles of differential centrifugation partially - purified preparations had a uv spectrum typical of a nucleoprotein (**Fig. 47**) with a A_{280}/A_{280} ratio of 1.6-1.8. A 1/5 dilution of this preparation produced uncountable numbers of lesions (**Fig. 48**) in ***C.quinoa*** and ***C. amaranticolor***. When diluted samples (1/20) stained in 2% *dodeca* - tungstophospheric acid (pH 6.5) were observed in the electron microscope, a moderate number of isometric particles with smooth round surfaces were seen. Moreover, some particles were penetrated by the stain (**Fig. 49**), suggesting that they were 'empty' shells (Hemida and Murant, 1989). Little host material was present in these preparations after two cycles of differential centrifugation. However, when the partially purified preparation was passed through the CPG column, and concentrated by high speed centrifugation, the virus recovery was poor, producing only five lesions when assayed in ***C. quinoa***. This poor recovery may have been related to the rather unstable nature of the virus (Table 63).

Fig. 47 The uv absorption spectrum of partially-purified PYFV-E from infected *N.clevelandii* (diluted to 1/100)

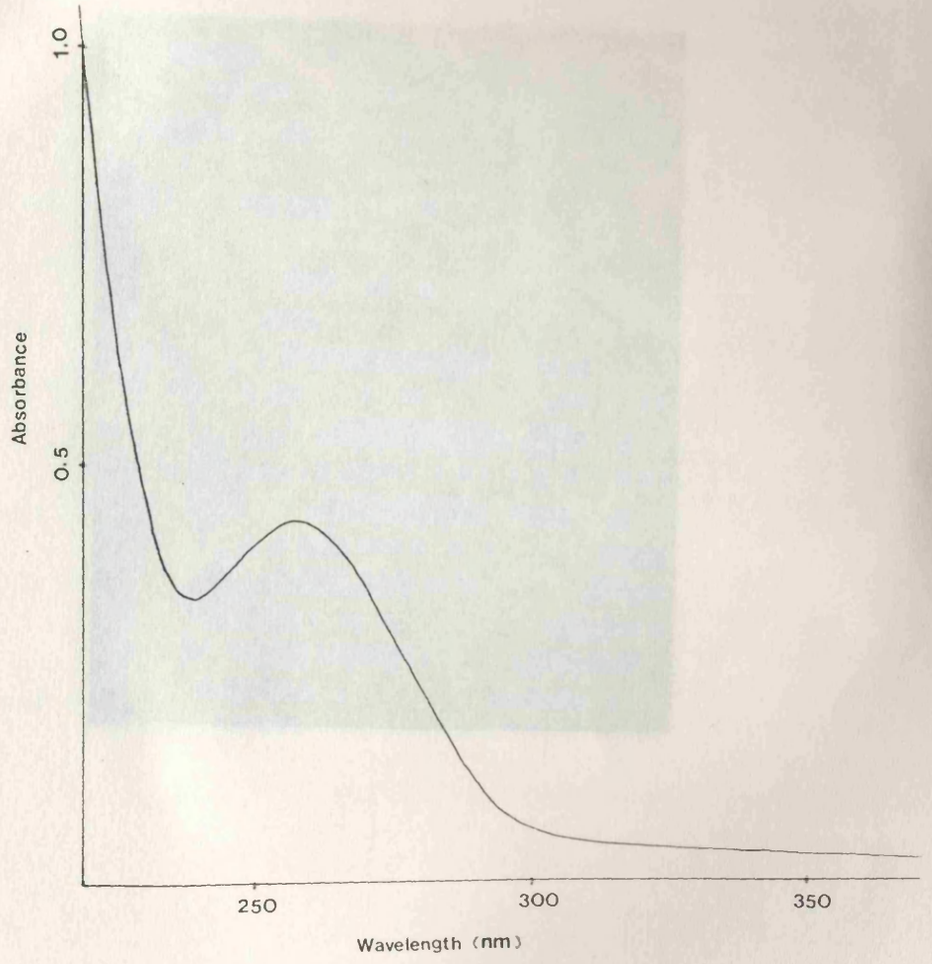
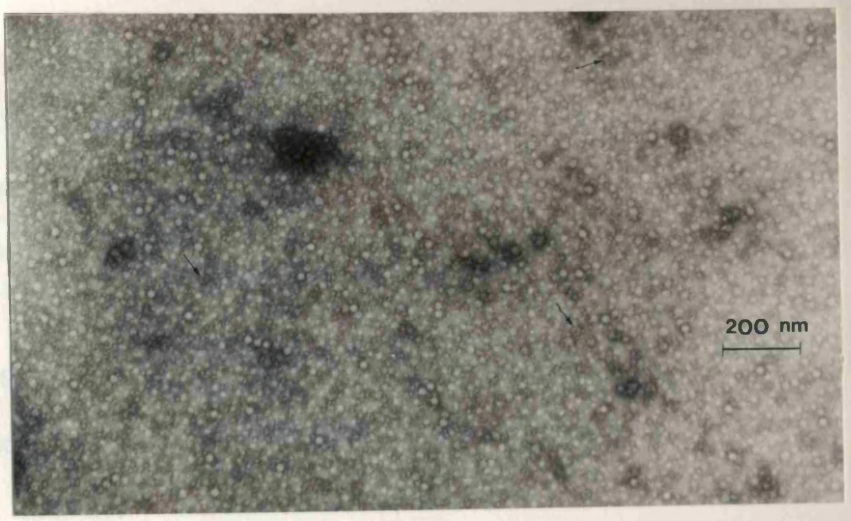


Fig. 48 Necrotic local lesions produced by partially-purified preparation (diluted to 1/5)of PYFV-E

Fig. 49 Electron micrograph showing particles of PYFV-E stained in 2% *dodeca* - tungstophosphoric acid,pH 6.5. 'Empty' shells arrowed.



9.5 Determination of relative molecular mass (Mr) of capsid protein of PYFV-E.

Partially-purified preparations of PYFV-E in 0.017M sodium phosphate buffer, pH 7 containing 1% SDS and 1% 2-mercaptoethanol were electrophoresed in 7.5% polyacrylamide tube gels.

Three bands were clearly resolved after staining with Coomassie blue (**Fig. 50**). The Mr (**Fig.51**) of these bands 36,29 and 22 ($x10^{-3}$) were comparable with published data of Hemida and Murant (1989) who reported a Mr values of 31,26 and 23 ($x10^{-3}$) for PYFV. Weber and Osborne(1969) have reported an accuracy of 10% with this method, so differences in Mr were within expected deviations. Poorly stained bands with high Mr were also present probably dimers or multimers of the major bands.

9.6 Serology

Partially-purified preparations of PYFV-E were used to produce an antiserum in a rabbit. The antiserum from each bleed was cross absorbed with a host protein preparation (Chapter 2.11a) and the titres determined in gel double-diffusion tests, using a two fold dilution series of the homologous antigen made in saline (0.85% Nacl).

With the purified antigen preparations it was found that the antiserum titre increased from first

Fig. 50 Electrophoretic mobility of PYFV-E in 7.5% SDS-polyacrylamide gels for 4h at 6mA/gel. (a) protein standards, (b and c), PYFV-E

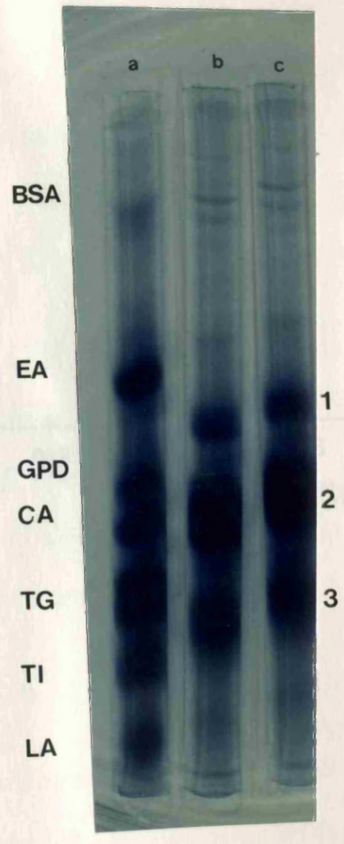
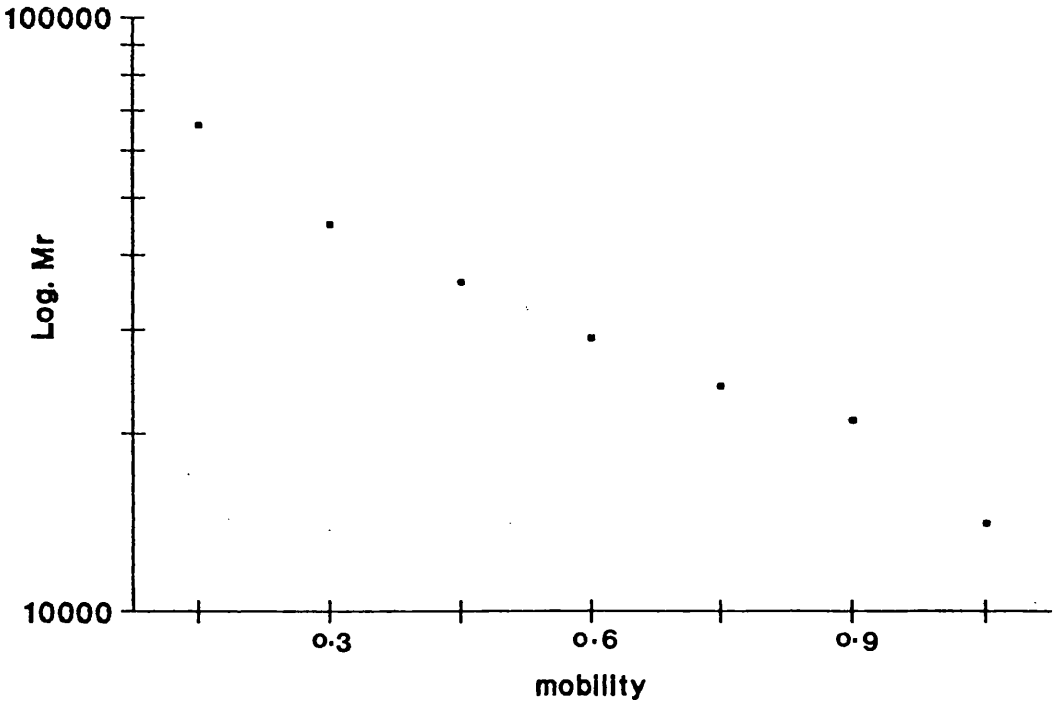


Fig. 51 Estimation of Mr of capsid protein of PYFV



x = 378 - 74.9 y

(1/8) to fifth bleed (1/128) and then decreased gradually. Crude sap from ***N. clevelandii*** was not a reliable source of antigen and did not always react with the antiserum, presumably because of low levels of virus. In most cases, therefore, partially purified preparations (either before or after high speed centrifugation, (Chapter 2.10) were used. Both crude sap when effective and partially purified preparation from healthy ***N. clevelandii*** gave precipitin lines down to 1/8 dilution of antiserum, indicating that the preparations used to prepare antiserum were not completely free of host proteins.

A virus isolated from ***Angelica archangelica*** in 1984 had properties similar to PYFV (Hicks, unpublished results). This isolate named AV1 was confirmed as PYFV in gel-diffusion tests, in the present study, using antiserum to PYFV.

Partial purification of AV1 and production of antiserum

Purification of AV1 was done as described for PYFV-E. Infected ***N. clevelandii*** leaves were used although this host appeared less susceptible to the angelica isolate than the parsnip isolate. Partially-purified AV1 was used to produce antiserum in a rabbit as described in Chapter 2.11a although only two injections, were given (one intravenous injection followed by one intramuscular injection a week later). The rabbit was bled once three weeks after the first injection. The

homologous titre obtained was 1/64.

In the gel-diffusion test with an undiluted antiserum to PYFV-E, both AV1 and PYFV-E produced single lines of precipitate which fused with each other suggesting that the antigens were closely related. Similarly, no spur formation was produced when undiluted antiserum to AV1 was used (**Fig .52**). The two PYFV isolates seemed to belong to P-121 serotype (parsnip strains) according to Murant's classification (Hemida and Murant, 1989). Moreover, this grouping was confirmed by the ability of PYFV-E and AV1 to infect ***N. clevelandii*** systemically, producing severe mottle. The strains which belong to serotype A-421 do not go systemic in this host (Murant and Goold, 1968).

9.7 Immunoelectrophoresis of PYFV-E and AV1

The immunoelectrophoretic mobility of partially purified preparations of AV1 and PYFV-E was compared in buffered agar gel, pH 8.2 Virus migration was located by placing homologous antiserum in troughs parallel to the path of electrophoretic migration and the distance travelled was measured from the origin of antiserum well to the end of the precipitin lines. the results are given in the **Table 64** and **Fig. 53.**

Fig 52 Reaction of PYFV isolates against antiserum to AV1 (centre well) a,c,f,e, PYFV-E; b,d,g, AV1 and h, healthy

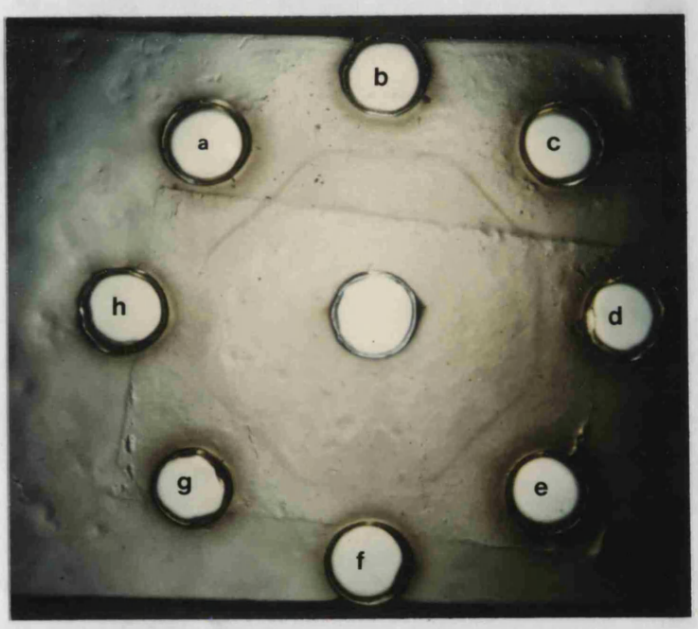


Fig. 53 Immunoelectrophorogram of PYFV-E and AV1 (electrophoresed for 3h at 100v in tris phosphate buffer, pH 8.6 and developed with antiserum to AV1

(a) AV1 (b) PYFV-E

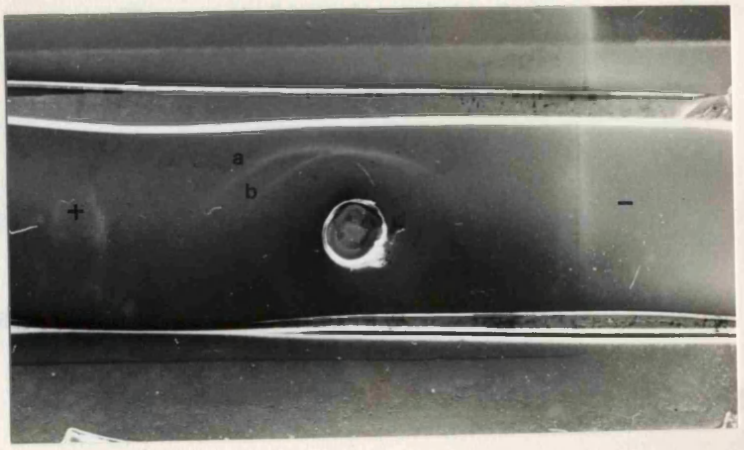


Table 64 Electrophoretic mobility of angelica and parsnip isolates of PYFV in TPE buffer (pH 8.2) electrophoresed for 2h.

Isolate	Distance travelled (mm)
PYFV-E	+7
AV1	+5

\+ Migrated towards anode

The results showed that both angelica and parsnip isolates of PYFV migrated towards anode, at a similar rate suggesting that they differed little in their electrophoretic mobility.

9.8a Detection of PYFV by indirect ELISA

ELISA was used to detect PYFV-E in infected hosts plants as it was found that the gel-diffusion test was not very sensitive particularly when crude sap was used. An indirect ELISA method using protein-A/ALP conjugate (Chapter 2.12) was therefore, used in this experiment to test the sensitivity of the technique in assaying PYFV-E. Optimum dilution of reagents were first determined using a 'checkerboard' format.

After carrying out a number of trial assays, an antigen dilution of 1/10 with 1/500 antiserum dilution were found to be the optimum combination to give a maximum discrimination between negative (healthy controls) and the positive samples; the results are shown

in Tables 65 and 66.

Table 65 ELISA readings (A_{410} values) of different dilutions of ***N.clevelandii*** sap (substrate incubated 4h at room temperature, and overnight at 4°C. D,infected; H,healthy)

Sap dilution (in Carbonate buffer pH 9.6)	A_{410} [a]As/500 D	[a]As/500 H	[a]As/1000 D	[a]As/1000 H
1/2	0.23	0.73	0.17	0.38
1/10	0.55	0.14	0.33	0.12
1/50	0.48	0.13	0.29	0.11

[a] Antiserum diluted in PBS-TPO

Table 66 A_{410} readings of ***N.clevelandii*** leaf sap with different antiserum dilutions (substrate incubated overnight at 4°C. D, infected, H,healthy)

Antiserum dilution	Sap dilution	[a]A_{410} D	[a]A_{410} H
1/500	1/10	0.42	0.14
1/1000	1/10	0.29	0.12
1/1500	1/10	0.29	0.12

[a]Mean of five replicates

These results with an antiserum dilution of 1/500 showed there was a high background effect (non-specific reaction) when concentrated (1/2) sap was used. But when the same antiserum dilution was used with an antigen dilution of 1/10, this gave at least 3-4 times greater A_{410} values (depending on the concentration of virus) than that of healthy samples. Similar results were

reported by Mowat and Dawson (1987) who used this technique to detect PYFV in infected *N. clevelandii* leaves. They reported an absorbance of 0.40 or 0.51 (depending on sap dilution) for infected sap and 0.14 for healthy sap, when 1/1000 antiserum dilution was used.

9.8b Effect of different extraction buffers on detection of PYFV-E

Different antigen extraction buffers namely, carbonate buffer (CB), CB+1% $NaSO_3$, CB+2% PVP, PBS-TPO and tap water were tested in this experiment. Infected leaves of *N. clevelandii* with PYFV-E and healthy leaves (as controls) were extracted in one of these buffers (1/10:w/v) and the indirect ELISA test done as described in chapter 2.12 . The results are given in Table 67.

Table 67 The A_{410} values of *N.clevelandii* leaf extracts infected with PYFV-E,extracted in different buffers (D,infected H, healthy)

Antigen Extraction buffer		[a]A_{410} D	H
CB	(9.6)	0.29	0.04
CB+1% $NaSO_3$	(9.4)	0.33	0.09
CB+2% PVP	(9.3)	0.43	0.09
Tap water	(7.1)	0.44	0.05
PBS-TPO	(6.7)	0.45	0.04

Figures in parenthesis are the pH values of buffers
a Mean of five replicates

These results showed that there was probably no marked difference between the buffers tested although the highest positive : negative ratio (11) was found with PBS-TPO extraction buffer. Tap water also showed a high positive : negative ratio (9) while carbonate buffer alone or with additives gave about 5-7 times greater values of A_{410} compared to healthy controls.

In the above experiment healthy samples were obtained from relatively young ***N. clevelandii*** plants (3-4 leaf stage seedlings). This could be one reason for the low non-specific reaction obtained in this experiment. When the experiment was repeated with older ***N. clevelandii*** plants selected for uniformity although the A_{410} values of healthy controls were (0.09-0.15) the results confirmed PBS-TPO and tap water were probably slightly better than other extraction media. The reason is not known although the lower pH values of these two extractors may possibly reduce the dissociation of virus that probably occurs at high pH. Water and PBS-TP (without 0.2% ovalbumin) were recommended as best extractors by several workers (Lommel ***et al***, 1982; Ehlers and Paul, 1984) although Mowat and Dawson (1987) reported that carbonate buffer was superior to the PBS-TP or water in their experiments.

In all future experiments with PYFV-E in protein -A ELISA tests, PBS-TPO was used as the extraction medium.

9.9 Back-inoculation of PYFV-E to parsnip

In order to satisfy Koch's postulates, seedlings of parsnips with 4-5 leaves were inoculated with partially-purified preparations of PYFV-E and the plants maintained at 18°C. A slight mottling in both inoculated and newly emerged leaves were noticed four weeks after inoculation in two out eight plants. All plants were back tested on *C. quinoa* and only those two plants with mild symptoms were found to be infected with PYFV.

Attempts to return a single lesion isolate of AV1 to healthy *Angelica archangelica* or *A. sylvestris* have so far failed.

9.10 Interaction between PYFV-E and SLRV

There are many examples of synergism between strains and even between different viruses (Matthews, 1981). Many different forms of non-genetic interaction between plant viruses in mixed infections have been reported (Atabekov and Dorokhov, 1984). The multiplication rate and/or distribution of one virus is often enhanced in the presence of another. A well known example is the stimulation of potato virus X replication, in co-infections with the unrelated potato virus Y, tobacco etch virus, tobacco mosaic virus, or cucumber mosaic virus (Atabekov and Dorokhov, 1984).

Because mixed infection between PYFV and SLRV in parsnip, though not proven, may occur naturally, an attempt was made to see if there was any interaction between these viruses in ***N. clevelandii*** and ***C. quinoa***. Small ***N. clevelandii*** plants selected for uniformity (4-5 leaf stage) were divided into two groups: one group was mechanically inoculated with PYFV-E , the other group of plants were inoculated with buffer only, on leaves pre-dusted with carborundum (600-mesh). All plants were grown at 18±1°C in a growth cabinet illuminated at 7,500 lux for 16h/day. Three days later,half of the plants in the group previously inoculated with PYFV-E were inoculated with SLRV-D (on the same leaves) and the other half were left uninoculated. At the same time, those plants previously rubbed with buffer only were also inoculated with SLRV-D. All plants were grown in a growth cabinet at 18°C for about three weeks until symptoms developed.

The results (**Fig.54a**) showed that plants inoculated with a mixture of PYFV-E and SLRV-D were more stunted and showed severe necrotic systemic infection compared to those infected with either virus alone. ***N. clevelandii*** infected with PYFV-E showed systemic chlorotic mottle with no apparent stunting (**Fig 54b**). Plants infected with SLRV-D alone showed no symptoms or a slight chlorotic mottle.

More severe systemic symptoms (necrosis

Fig. 54a *N.clevelandii* infected with PYFV-E and SLRV-D

Fig. 54b *N.clevelandii* infected with PYFV-E alone.

with leaf distortion) were seen in doubly infected plants when the challenge inoculation was made seven rather than three days after the first inoculation with PYFV-E. In general, symptom expression in doubly-infected plants was less severe when the plants inoculated were older, presumably because they were more resistant to infection.

N. clevelandii plants became infected with both viruses when both viruses were inoculated together. A mixed infection was also established in ***N. clevelandii*** when SLRV-D was inoculated prior to PYFV-E although it resulted in less severe symptom expression than when SLRV-D was inoculated after PYFV-E.

To determine whether symptom severity in plants with mixed infection resulted in an increased concentration of SLRV, leaf samples from both singly and doubly infected plants harvested 15-18 days after inoculation, were tested by DAS-ELISA. The results are given in **Table 68**.

Table 68 A_{410} readings (mean of four replicates) of leaf extracts from ***N. clevelandii*** in a DAS-ELISA experiment using an antiserum to SLRV-D (substrate incubated for 45 min at room temperature,H,healthy)

Dilution	SLRV-D	PYFV-E+SLRV-D	H
1:25	0.33	0.33	0.09
1:125	0.22	0.27	0.09
1:625	0.12	0.17	0.08

The results showed even with the lowest dilution (1/25) the absorbance values were relatively low (0.33) for both singly- and doubly- infected leaves, indicating low concentrations of SLRV. Slightly larger A_{410} value (0.27) was obtained for SLRV-D in mixed infection compared to that of singly infected plants, when a sap dilutions of 1/125 and 1/625 were used, but these were probably not significant.

The concentration of PYFV-E in doubly and singly - infected plants was determined by assaying systemically infected ***N. clevelandii*** leaves on ***C. quinoa*** Based on lesion counts it was observed that the concentration of PYFV-E had been significantly increased in the presence of SLRV-D. However, when leaf samples from the same ***N. clevelandii*** plants were tested in a protein-A ELISA no differences were found in A_{410} values between the two types of infection. It was possible that the indirect ELISA technique does not reliably measure virus concentrations, especially in mixed virus samples where competition for binding sites may occur if the concentration of one virus greatly exceeds that of the other (Mowat and Dowson,1987). However , more work is needed and analysis of tissue for dsRNA may give a clearer picture of the relative concentrations of virus in mixed infections.

When this experiment was repeated using ***C. quinoa*** plants instead of ***N. clevelandii*** , similar results

were obtained with respect to symptom production. Thus, when young seedlings of *C. quinoa* were inoculated with PYFV-E and SLRV-D, severe systemic necrotic symptoms were produced which usually led to plant death. Plants inoculated with either PYFV-E or SLRV-D normally showed mild systemic and/or local infection but plants were not killed.

In further experiments, a similar type of synergism was found between the nepovirus, raspberry ringspot virus (an isolate from *Forsythia*), and PYFV-E. Symptoms in singly and doubly infected *N. clevelandii* are shown in **Figs 55a and b**.

9.11 Seed transmission of PYFV-E

Those *N. clevelandii* plants doubly inoculated with PYFV-E and SLRV-D in the previous section were transferred to a glasshouse at 20-24°C after flowering, and left to set seed. When the undehisced anthers ground in inoculation buffer were assayed on *C. quinoa* plants, papery necrotic local lesions characteristic of PYFV-E were produced, followed by a severe systemic mottle which was more characteristic of SLRV and PYFV in a mixed infection. This observation indicated that PYFV as well as SLRV might occur in the pollen. An experiment was then done to see if PYFV was seed-borne in *N. clevelandii*.

Fig. 55a *N. clevelandii* infected with RRV and PYFV

Fig. 55b *N.clevelandii* infected with RRV alone

Seeds collected from doubly infected *N. clevelandii* plants were germinated and about 20% of the seedlings were found to be stunted with a severe loss in vigour. When seedlings were individually assayed on *C. quinoa*, about 75% of the test plants showed a systemic mottle chracteristic of SLRV-D. A further 20% showed local chlorotic spots followed by a severe systemic yellowing and plant death. The same seedlings produced symptoms typical of PYFV-E (or SLRV-D), when assayed on coriander. Gel-diffusion tests confirmed SLRV-D in *N. clevelandii* seedlings, but not PYFV-E. However, when these seedlings were bigger (5-7g fresh weight), each seedling was partially-purified and the preparations were found to contain PYFV-E when tested in gel-diffusion tests. Thus,20% of the seedlings were infected with both SLRV and PYFV.

In further experiments, the same seed batch was germinated in an insect-protected glasshouse and seedlings tested in indirect ELISA with homologous antiserum to PYFV-E. Four batches of 30 individuals were tested in four different plates, with negative (healthy) and positive (PYFV-E infected) controls in each plate. The results of one batch is given in **Table 69**.

Table 69 The absorbance values of extracts of *N.clevelandii* seedlings raised from seeds of doubly-infected plants (PYFV-E antiserum substrate 48 h at 4 C).

Seedling No.	A_{410}
1	[a]0.40
2	0.13
3	0.17
4	0.24
5	[a]0.33
6	0.27
7	0.18
8	[a]0.33
9	0.14
10	0.22
11	0.20
12	0.10
13	0.13
14	0.15
15	0.12
16	0.12
17	[a]0.38
18	0.11
19	0.10
20	0.18
21	0.18
22	[a]0.40
23	0.10
24	0.11
25	0.14
26	0.16
27	0.11
29	0.11
30	0.15
-ve control	0.15
+ve control	0.88

[a] considered as positive for PYFV-E

The results showed at least 17% of these *N. clevelandii* seeds were carrying PVFY-E. Those plants which were positive for PYFV-E (ie; plant no. 1,5,8 , 17 and 22) were grown further,partially-purified and, tested in gel-diffusion tests with antiserum to PYFV-E. All

samples gave precipitin lines confirming the presence of PYFV-E. However, these results also indicated that although PYFV-E was seed transmitted in ***N.clevelandii***, the concentration of virus in the seeds seemed to be very low.

Having confirmed that PYFV-E was transmitted in the seeds of ***N. clevelandii*** plants infected with SLRV-D, the next experiment was to see if PYFV-E was seed transmitted in the absence of SLRV.

In this experiment, eight, uniform plants of ***N. clevelandii*** were inoculated with PYFV-E and four of them also inoculated with SLRV-D, a week after the first inoculation. All plants were grown in a growth cabinet at 18°C and transferred to a glasshouse at 20-24°C after flowering and left to set seed.

Seed collected from plants infected with PYFV-E alone were germinated and about 200 seedlings individually assayed for virus by indirect ELISA (about 30 samples per plate). Both negative (healthy) and positive controls (PYFV-E infected) were included in all plates. Those from plants infected with both viruses were also assayed for PYFV-E by indirect ELISA and the same seedlings for SLRV-D by DAS-ELISA.

The results showed that the seeds from plants infected with PYFV-E alone were carrying the virus (10-30%) in the absence of SLRV-D. The absorbance of

those samples considered as positive for PYFV-E ranged from 0.38-0.55 whereas the healthy controls were within the range of 0.12-0.15 while positive controls were 0.77-0.91 (48h substrate incubation at 4°C). Moreover, almost the same percentage from doubly - infected plants were found to be carrying PYFV-E in seeds which were not infected with SLRV-D. The SLRV was found in about 70-85% progeny seedlings.

Chapter 10 DISCUSSION

Strawberry latent ringspot virus (SLRV) was isolated from field-grown parsnips of cv White Gem by inoculation of buffered leaf extracts to *C. quinoa* . In previous reports (Cooper, 1981; Hicks *et al.,* 1986), infection of parsnips was not usually associated with the production of conspicuous symptoms. In the present study, however, parsnips infected with SLRV generally showed virus-like symptoms although similar symptoms were also present on parsnips not apparently infected with SLRV (or any other readily sap-transmissible virus). Symptom severity varied from severe on plants infected with the isolate SLRV-D and I to mild on plants infected with SLRV-F, K, or L (Table 2). Furthermore, flowering and seed setting of parsnips infected with isolates D and I were delayed by 6-7 weeks compared to healthy plants or plants infected with other isolates. Although the evidence was circumstantial only , it is possible that with these isolates infection had decreased the germination rate and growth in the field during the first growing season. Parsnips raised (under glass) from infected seed usually showed premature flowering.

Strawberry latent ringspot virus is typically symptomless in many hosts (Lister, 1964) and first reports of SLRV in parsnip (Cooper, 1981, Hicks *et al.,* 1986) tended to support this. In the present work, although SLRV was associated with foliage symptoms in the field, seedlings

raised (under glass) from infected seed at temperatures of 20-28°C with supplementary lighting, rarely showed symptoms. However, when infected seed was germinated and grown at constant temperature and light (18°C/7,500 lux) most of the seedlings showed a general chlorosis, or a chlorotic mottle with numerous chlorotic spots on the older leaves. Within three or four months a severe chlorotic mottle developed on both young and older leaves. Seedlings from healthy seed grown under the same conditions never showed symptoms.

Cooper (1981) had noted a similar mottling on parsnip seedlings grown in the field, which was associated with infection by SLRV. Moreover, the symptoms which were noted in July (1977) had disappeared completely by early August and led to his suggestion that the low temperatures in the cold wet spring of 1977 induced the transient foliage symptoms. The present study, therefore, supports these observations; the decline in symptom expression possibly being due to the partial supression of virus synthesis during the high temperatures, which lowered virus concentration (Kassanis, 1957; Pound, 1945; Pound and Weathers, 1953). However, there is not always a simple relationship between virus concentration and symptom expression (Matthews, 1981).

In some hosts with SLRV there also appears to be a delay between infection and symptom expression. Thus in glasshouse experiments Walkey and Mitchell (1969) reported an incubation period of about six months before celery plants

inoculated with SLRV develped 'strap leaf' symptoms. In the field, diseased celery plants were not observed before the end of September. Lister (1964) noted a similar delay between inoculation and symptom production for SLRV in ***Petunia***. If nematodes are involved in transmission there may be a delay between the infection of the roots and the production of symptoms in the foliage (Thresh, 1974). In these examples the delays were probably due to delays in virus replication or movement. While these factors may have contributed to the enhanced symptom expression in older parsnips, environmental factors were also involved in the present study. Thus, when parsnip plants were repotted in Levington Compost or fertiliser added, symptoms gradually disappeared indicating that symptom production in these plants was associated with nutrient stress. However, the effect of plant nutrition on virus multiplication is highly variable and symptom expression may be enhanced or reduced according to the host, nutrient and virus involved (Matthews, 1981). Thus, Broadbent and Tinsley (1953) found that nitrogen increased the rate at which cauliflower mosaic symptoms developed in cauliflowers; it also increased their severity, whereas phosphate decreased them and potash had no significant effect. It would be worth studying this effect further in parsnip, looking at individual macro and minor elements because it raises the possibility, that a reduction in some specific element may play a part in the induction of virus disease symptoms. To exclude the possibility of mixed infection, virus-free parsnips infected

with pure virus would have to be used.

In the present study, apparently healthy parsnip seedlings were infected with single lesion isolates of SLRV which developed chlorotic symptoms similar to those seen on seedlings raised from infected seed. Viruses apparently identical to the original isolates were obtained from these plants strongly implicating them in the observed disease according to criteria given by Bos (1983).

Although symptoms may have been due to non-viral causes, the failure to isolate virus from some parsnips with virus-like symptoms may have been due to either low virus concentrations in leaf tissue or the presence of inhibitors in the inocula. Matthews (1981) has reported that high amounts of leaf inhibitors can prevent sap transmission of a virus. In the present study, healthy parsnip leaf sap produced a 73% inhibiton of local lesion formation by tobacco necrosis virus (TNV) in ***Phaseolus vulgaris*** when present in the virus inoculum. However, only 42% inhibition was found when healthy root sap extracts were mixed with TNV inoculum. These results suggest that in future surveys, root as well as leaf inocula should be sampled where they are to be assayed for infectivity. Root tissue was better than leaves for the isolation of clover yellow mosaic virus from pea (Ford, 1973).

Relatively low virus concentration in parsnip leaves, was indicated by experiments in which antigen concentrations were measured by DAS-ELISA (Chapter 4. 6c).

Dilution end-points for antigen in parsnip leaves generally reached only 1/25 sap dilutions compared to at least 1/192,000 for SLRV in *C. quinoa* . Low levels of virus replication were also suggested by consistently reduced band intensities of dsRNA extracted from parsnip leaves compared to *C. quinoa* leaves (Chapter 4.7c). Higher concentrations of SLRV in parsnip root tissues were indicated by higher A_{410} values in DAS-ELISA and by higher dsRNA band intensities. It was probably coincidence, however, that these higher virus levels were associated with lower levels of substances inhibitory to virus infection by sap transmission.

The isolates obtained from parsnip when observed over three or four weeks in the glasshouse, differed somewhat in their virulence on *C. quinoa* . There was little evidence, however, of a correlation with symptoms in parsnips although SLRV-I was associated with more severe symptoms in both hosts. The isolates from parsnip could, broadly, be placed into three groups in their reaction on *C. quinoa* : 'mild' (SLRV-R)which induced mild systemic chlorotic mottle and slight reduction in vigour, 'severe' (SLRV-I) which induced systemic mottle with extensive apical necrosis, and 'intermediate' (SLRV-D) which induced symptoms of intermediate severity. Grouping of nepovirus isolates on the basis of virulence in particular hosts is common. Thus, Hicks (1979) identified 'mild' and 'severe' isolates from rose according to their reaction in *C. amaranticolor* . The same classification was made for isolates from rhubarb (Tomlinson and

Walkey,1967) and strawberry (Lister, 1970).

The host range of parsnip SLRV isolates was moderately wide at least compared to a previous limited study of SLRV in parsnip (Smith, 1986). Thus, both cucumber and ***N. tabacum*** cv Xanthi were not susceptible to his isolates, whereas all three isolates studied (SLRV-D,R, and I), in the present work, infected these species. Apart from differences in virulence between D, R and I in ***C . quinoa*** , these isolates differed in their ability to infect umbelliferous hosts. Thus, although SLRV-D infected carrot and dill, SLRV-I did not infect either; both isolates, however, infected coriander. Isolate R infected celery (but with difficulty) while D and I did not infect this host in repeated attempts. Natural infection with SLRV has been reported for celery (Walkey and Mitchell, 1969), so cultivars may differ in their susceptibility. There are also differences in pathogenicity between isolates. For example, only three out of eight isolates of SLRV from rose infected celery (Hicks, 1979). In the present study differences in host susceptibility could not be related to differences in inoculum strength. For example, the DEP of SLRV-R was the highest of the isolates tested but this isolate produced the mildest symptoms in ***C. quinoa***. Similarly, isolate R ('mild') induced severe symptoms in cucumber whereas the other two isolates produced only mild symptoms. This emphasises the arbitrary nature of the virulence groupings which depend on the host used for the classification.

The main differences in host range and reaction between isolates of SLRV from parsnip (Smith, 1986) and strawberry (Lister, 1964) are given in the Appendix (Table 2).

The environmental conditions under which plants are grown before inoculation, at the time of inoculation, and during the development of symptoms can have a profound effect on the course of infection (Matthews,1981). Light and temperature are the main factors to be considered. In the present study, the effects of temperature on the susceptibility of plants to SLRV were variable. Relatively high temperatures (short periods) before inoculation decreased the susceptibility of ***C.quinoa*** and cucumber to infection. This was in contrast to the work of Kassanis (1952) who showed that with several host/virus combinations (though not SLRV or cucumber), a period of high temperature increased susceptibility. However, the findings were in general agreement with Lindner ***et al.***,(1959) and Hicks and Frost (1979) who reported decreased susceptibility of cucumbers grown at high temperatures. The mechanism of this effect is not known and while it is possible the resistance of cells to infection at these temperatures was increased, the effect may be related to differences in ease of wounding, and cuticle thickness which may increase at high temperatures (Matthews, 1981).

The effect of short periods (4h) at 4°C were less clear cut. In the present work, the effects of pre-chilling were not significant although total lesion numbers

increased by 30%.(compared to plants at 20°C). Lindner *et al* (1959) reported decreased susceptibility of cucumbers with pre-chilling at 2° C although Bokx (1972) found increased susceptibility of ***Physalis floridana*** to potato virus Y when plants were kept at 4-10°C for a short time before inoculation. Critical comparisons between different authors, however, are difficult because different conditions and host/virus combinations have been used. The effect of pre-inoculation darkening was also variable. Thus, while 24 h dark period increased the susceptibility of 5-6 week old ***C. quinoa*** (to SLRV),little effect was seen on ***C. quinoa*** seedlings or in cucumber. Extending the dark period to 60 h significantly reduced the susceptibility of cucumber to infection. Lindner ***et al.,*** (1959) found that a 24 h dark period was necessary for maximum susceptibility of cucumber to tobacco mosaic virus (TMV). Similarly, Bawden and Roberts (1948) found that a dark period before inoculation increased the susceptibility of ***N. glutinosa*** to TMV. Many plant virologists routinely give plants a dark treatment before inoculation, but the above results suggest that more care is needed in its use.

High temperatures after inoculation often reduce symptoms (Walkey, 1985). In the present study, a temperature of 33°C immediately after inoculation reduced infection of *C.quinoa.* by SLRV although temperatures of 4 and 26 °C did not. Prolonged exposure to 28 °C reduced the concentration of virus as determined by ELISA. Two to four weeks at 33°C resulted in a failure to detect virus in the

foliage, although after returning to normal temperatures (18° C) virus was occasionally detected in the roots and less often in the foliage. According to Matthews (1981) growth at high temperature may interfere with the the stability of enzymes essential for virus replication and may inhibit virus assembly.

Pink and Walkey (1984) found that when CMV-infected marrow seedlings were grown at 25°C symptoms were few, but the symptoms greatly increased when plants were grown at 20° or 15° C. Similarly, in the present work, symptoms were supressed in cucumber plants grown at 26°C after inoculation compared to those grown at 18°C.

In the above experiments it was not usually possible to differentiate between effects of temperature on virus replication and those on symptom expression. Evidence from the analysis of dsRNA extracted from plants grown at 26°C, however, strongly suggested that virus replication was being inhibited compared to plants grown at 18°C. The rate of multiplication (as shown by maximum yields of dsRNA), however, was faster at 26°C. These results are in general agreement with those of Lebeurier and Hirth (1966) who showed that levels of TMV in tobacco leaf discs (determined serologically) peaked sooner at 28°C and reached lower overall concentrations than in discs kept at 20°C.

For viruses that are difficult to purify, careful manipulation of the environmental conditions under

which plants are grown before and after inoculation can improve final yields (e.g. Hicks, 1979). In the present study, ***C. quinoa*** was found to give satisfactory yields of SLRV- ~1mg per 100 g leaf material- when grown at 18-20°C and harvested about 8-12 days after inoculation. Clarification with 8.5% (v/v) butan-1-ol, used here for SLRV-D and R, followed by concentration with polyethylene glycol precipitation or differential centrifugation were reported to be useful procedures for nepovirus purification (Murant, 1981)

Further purification was accomplished by passing the virus through a column of controlled pore glass (Barton, 1977). Higher than expected absorbance values (260 nm) and 260:280 ratios were frequently obtained for preparations clarified with butan-1-ol, presumably due to some host component absorbing strongly at 260nm. This problem has been reported before for this solvent (Hollings, 1974; Tomlinson ***et al.***,1959) but was eliminated by a second passage through the column. Preparations examined in the electron microscope showed isometric particles about 26-28 nm in diameter often with hexagonal outlines. Most particles in the butan-1-ol preparations were unpenetrated by negative stain. However, particles after clarification with hydrated calcium phosphate appeared mostly 'empty' (penetrated by stain), indicating that the full particles had been adsorbed to the gel. This would be consistent with the poor infectivity of these preparations.

Antisera to two SLRV isolates were produced with homologous titres of 1/128 (R) and 1/256 (D). In gel-diffusion tests with each other and with an isolate of SLRV from ***Caryoptreris*** , reciprocal tests showed that all isolates were similar serologically with serological differentiation indices of only 1-2. Cross adsorption studies would be needed to establish if the isolates were identical or only closely related serologically. The 'spur' formation seen between parsnip and ***Caryopteris*** antigens against ***Caryopteris*** antiserum, disappeared when antiserum was diluted and may, therefore, have been the 'false spurs' due to uneven concentrations of reactants (Crowle, 1973). Some evidence that the parsnip isolates were different from SLRV-C and from each other came from their distinct electrophoretic mobilities, although differences were most clear cut between SLRV-D and SLRV-C. Walkey *et al* (1972) used a similar method to differentiate between strains of cherry leaf roll virus. Furthermore, as with SLRV , their isolates produced two distinct precipitin lines in immunoelectrophoresis but only one line in gel diffusion.

Many SLRV isolates reported from a wide range of hosts have been found to be serologically related except for one isolate from cow parsley (SLRV-P). This isolate was reported to be distantly related to an English isolate (strain E) of SLRV from celery when tested against antiserum to SLRV-T39 (Henson and Campbell,1979).

The DAS-ELISA was found to be useful for the detection of SLRV isolates in many test hosts including ***N. rustica*** ,and ***C. amaranticolor*** and particularly in parsnip, where gel-diffusion failed.

The analysis of dsRNA from plant tissues is a useful tool for detection and possible identification of plant viruses (Dodds ***et al.***, 1983). The dsRNAs of SLRV were successfully extracted from several hosts, including parsnip, while dsRNA was not found in extracts from healthy hosts.

About 5 - 10g of young parsnip leaves infected with SLRV was sufficient to give detectable amounts of dsRNA, although the first major band was sometimes hardly detectable. If detection only is required the use of dsRNA analysis may be a feasible alternative to sap transmission of SLRV or any other virus from parsnip leaves, which were found to contain high amount of inhibitors.

No major differences were found in the pattern of dsRNA segments extracted from different hosts although 'minor' species (possibly sub-genomic) were present in preparations from ***C. quinoa*** , ***C. amaranticolor,*** and sometimes ***N. clevelandii.*** It was interesting that these minor bands were not found in extracts of plants, like parsnip, which were mostly symptomless.

The faint nature of dsRNA-1 from parsnip leaf tissue was unexpected, and contrasted with that found in species such as *C. quinoa* and *C. amaranticolor* where it was noticably bright. Possibly RNA-1 was responsible for the symptom production in *Chenopodium* species. In support of this is evidence that in *C. quinoa* and other herbaceous hosts, infected with raspberry ringspot virus , symptom severity was determined by RNA-1 (Harrison *et al.*, 1974). It would be interesting in future experiments to investigate whether the same function was determined by RNA-1 of SLRV.

The SLRV infection of all three isolates had a marked effect on the yield on many experimental hosts. Root yield of *Beta vulgaris* cv. Globe was significantly ($P<0.05$) reduced by infection with the 'mild' isolate, SLRV-R. The 'intermediate' isolate (SLRV-D) significantly ($P<0.05$) decreased the seed yield of *Spinacea oleracea* . The seed yield of the umbelliferous crop, *Coriandrum sativum* was also significantly affected by infection with either isolate I or D. No significant effect on the root yield of parsnip infected naturally with SLRV-I or D was found.

Unlike other experimental host tested, parsnip had to be grown in the glasshouse for about 9-10 months before the roots were big enough to harvest. During these experiments it was found that the glasshouse daytime temperature frequently reached 30-32°C. It was possible, therefore, that the SLRV concentration may have sharply declined as observed by the

inability to detect SLRV in the foliage of some plants during the summer. The low levels of SLRV may have contributed to the non-significant effect of infection on root yield.

Hicks ***et al*** (1986) found a significant difference in the seed weight of parsnip naturally-infected by a 'severe' isolate of SLRV, compared to that of healthy seeds. In their studies, parsnips infected by a 'mild' isolate of SLRV, however, produced similar mean seed weights compared to apparently virus-free seeds. No differences were found in the mean seed weight of parsnips infected with a 'mild' (SLRV-D) or 'severe' isolate (SLRV-I) in the present study, although seeds from infected plants were apparently smaller and more shrivelled than those from healthy plants.

All three SLRV isolates (SLRV-D, I and R) were readily transmitted through seeds although the percentage transmission varied with the host species and the temperature at which the seeds were produced. No differences were found in the seed transmissibility of the isolates in a range of host plants (Table 37). Hicks ***et al*** (1986) also did not observe a difference in seed transmission between their isolates. In contrast, Schmelzer (1969) found that seed transmission of SLRV from various woody hosts, through ***C. quinoa*** seeds, varied with the isolates. The high rate of seed transmission of SLRV in the host plants tested, signified its importance in the ecology of the virus. The importance of seed transmission in the ecology of nematode-borne viruses was discussed by Lister

and Murant, (1967) and Murant (1983). All virus-vector nematodes are ectoparasitic and migrate slowly through soils. The rate of migration of ***Xiphinema diversicaudatum*** Micol. was calculated to be only 30 cm per year over a period of 75 years (Harrison and Winslow, 1961). Compared to seed transmission therefore, vector transmission probably plays only a minor role in the dissemination of nepoviruses (Murant, 1983).

Generally, all three isolates significantly reduced the percentage germination of seeds in both natural and experimental hosts, although, no differences were found between isolates. Hicks ***et al.***, (1986), however, reported a significant difference in percentage germination between their isolates of SLRV from parsnip, and in mean seed weights.

The effect of SLRV on vigour of the seedlings raised from infected seeds varied with the host species, environmental factors and the isolates. Although, seedlings infected with nepovirus from seeds are usually symptomless and appear to be already in the 'recovered' condition (Tuite, 1960; Lister and Murant, 1967), some nepovirus / host combinations do not show this characteristic. In the present study, seedlings of ***C. quinoa*** infected with each of the three isolates showed a mild chlorotic mottling and a growth rate which was lower than that of non-infected healthy seedlings. Coriander seedlings infected with SLRV-I showed a marked loss of vigor

and severe necrosis particularly at lower temperature (18°C) when they were easily distinguished from non-infected seedlings. On the other hand, coriander infected with SLRV-D showed no apparent loss of vigour and only mild symptoms and would, therefore, needed to be assayed to detect virus.

In *C. quinoa* the seed transmission of SLRV was 100 % at 18°C, 28% at 28°C and about 0% at 33°C whereas in *N.rustica* it was 45 % at 18°C and 23% at 33°C (Table 43). Similarly, another nepovirus, cherry leaf roll was 100% and 0% transmitted through seeds collected from *N. rustica* plants grown at 20°C and 30°C respectively (Cooper,1976 cited by Walkey, 1985). Crowley (1969) found that southern bean mosaic virus was seed tranmitted in 95% of seeds harvested from plants grown at 16-20°C, but only 55% of seeds from those grown at 28-30°C. On the other hand, Hanada and Harrison (1977) reported that temperatures of about 14°C reduced the seed transmission of SLRV in chickweed (0%) compared with seed produced at 18°C (24%) or 22°C (29%).

It is evident that relatively low or high temperatures reduced the seed transmission of SLRV. As Hanada and Harrison (1977) suggested,lack of transmission of SLRV at lower temperatures (14°C) may be partly due to the delay in systemic infection of mother plant. In the present work, the high temperature may have reduced the SLRV concentration in the host meristems (Walkey, 1985) and this reduced the amount of

virus transmitted in the seeds.

Several nepoviruses are reported to invade the embryos of infected seeds (Athow and Bancroft, 1959; Crowley, 1959 ; Lister and Murant, 1967). In infected parsnips and *C. quinoa* seeds, SLRV was recovered from embryos, but not from testas (Hicks *et al.*, 1986). This was also found in the present study; SLRV,therefore, can be regarded as a true seed transmitted virus, distinct from viruses like tobacco mosaic virus which occur mainly in or on the seed coat (Walkey, 1985). According to Crowley (1959), infection before flowering and before the female gametes were formed was essential for embryo transmission of southern bean mosaic virus in bean seeds. In the present work, the age of plants at infection did not have a marked effect on seed transmission of SLRV. Similar levels of seed transmission of SLRV were observed in *C. quinoa* whether plants which were inoculated seven or 14 weeks after sowing. It was apparent that SLRV could move efficiently from the inoculated leaf to the apical part , even after plants had flowered and infection of the megaspore mother cell at an early stage before flowering, was not necessary. However, the stage of embryo development at the time of inoculation was not known. More work is needed to identify critical factors in embryo infection by SLRV.

SLRV was successfully isolated from pollen of infected parsnip plants and many other test plants . It has been well established that the female gametes can be infected

through pollination by infected pollen (Schippers, 1963). Evidence for pollen transmission has been found by several workers. For example, Medina and Grogan (1961) found bean common mosaic virus transmission in bean seed was high if healthy female parents were pollinated with infected pollen although it was higher when both mother plant and the pollen donor were infected. Pollen transmission has also been reported for several nepoviruses, including cherry leaf roll in birch (Cooper, 1976) and tobacco ringspot virus in soybean (Yang and Hamilton, 1974).

The present study showed, apparently for the first time, that SLRV was pollen-borne (in cucumber) and that seed could become infected via infected pollen. Transmission of SLRV in seeds of healthy plants pollinated with infected pollen was 40 % compared to 90-100% when the both male and female parents were infected. Similarly Cooper (1976) reported that fewer birch seeds were infected with cherry leaf roll virus when female flowers on infected trees were fertilized with healthy pollen than when virus-carrying pollen was applied to healthy trees. In contrast, the percentage of seeds infected with SLRV was slightly higher (40-60%) when infected female flowers were pollinated with healthy pollen than when healthy plants were pollinated with infected pollen.

There was no evidence that parts of the plant other than seed were infected through fertilization of healthy cucumber with infected pollen. However, some viruses such as

the ilarviruses prunus necrotic ringspot and prunus dwarf viruses in cherry (George and Davidson,1963) and raspberry bushy dwarf virus in raspberry (Cadman,1963) were reported to be spread in this form. Further tests would be needed to establish whether a recipient plant become infected by pollination with SLRV-infected pollen under the cooler temperatures found in the field.

Yang and Hamilton (1974) reported a poor germination rate and a low percentage germination of soybean pollen infected with tobacco ringspot virus and suggested infected pollen might have a poor competitive ability and be of little importance in the ecology of the virus. Similar observation were made by Lister and Murant (1967) for raspberry pollen infected by raspberry ringspot virus. With SLRV, the effect of infection on the percentage germination and rate of germination of pollen, and the length of pollen tubes, varied with the host. In tobacco cv. Xanthi, pollen germination was not affected by infection. In contrast, pollen grains of ***N.clevelandii*** infected with SLRV showed a significant reduction in germination and also poor growth rate.

Strawberry latent ringspot virus was successfully returned to healthy parsnip using mechanical, nematode and graft transnmission techniques, in partial fulfilment of Koch's postulates. Parsnip plants could not be infected mechanically with extracts from parsnip leaves presumably because of low virus concentration and/or the

presence of inhibitors. Root inocula were effective but post-inoclation temperature was critical with infection inhibited at 26°C. However, when plants were infected by nematodes, transmission was more efficient when inoculation feeds were at 26°C rather than 18°C. More vigorous root growth at 26°C may have increased the surfaces available for inoculation or may have increased vector activity and probing (Boag, 1978). Alternatively, the higher moisture status of pots at 18°C may have produced more anaerobic conditions which may be less suitable for the vector. Cooper and MacCallum (1984) reported reduced numbers of trichodorids, under similar conditions.

In general, ***C. quinoa*** was more susceptible than parsnips to infection by nematodes. Trudgill and Brown (1978) also found ***C.quinoa*** to be a favourable host for transmission of SLRV by ***X. diversicaudatum***. With parsnips, recently germinated seedlings were found to be more susceptible to nematode infection than those young plants at the 4-5 leaf stage. Harrison and Winslow (1961) reported similar findings for the infection of pea seedlings by ***Arabis*** mosaic virus.

Unlike many test plants, it was not possible to infect parsnip leaves with purified preparations of SLRV, and no other source of inocula except buffered parsnip root sap was successful in infecting parsnip leaves manually. Although the reason was not determined it was possible chemicals in the parsnip leaf released during abrasion, or present in the cells, interfered with virus establishment. Infected parsnip plants

had higher concentrations of SLRV in roots than in leaves suggesting root cells may be more susceptible sites for virus multiplication than leaf cells. Furthermore, mechanical transmission by inoculation of roots has been achieved for some viruses (e.g. Moline and Ford, 1974) and would be worth trying for parsnips.

Tissue culture techniques, particularly in combination with thermotherapy or chemotherapy have been used for the production of virus-free plants (Walkey,1985). In the present study, heat treatment of ***in vitro*** grown seed cultures of ***C.quinoa*** ,***N.rustica*** and ***P.sativa*** infected with SLRV, resulted in the elimination of virus as judged by infectivity assay and DAS ELISA. The temperature required for the inactivation and eradication of SLRV were similar to those needed to eliminate cherry leaf roll virus (Cooper and Walkey, 1978) cucumber mosaic virus and alfalfa mosaic virus in ***N.rustica*** seed/embryo cultures (Walkey 1976). For these viruses, a temperature of 32°C appeared to be critical for virus eradication. However, the duration of the heat treatment required for a total elimination of the virus varied with the different host/virus combinations. For example Cooper and Walkey, (1978) suggested a period of 40 days treatment at 32°C would be required for the total eradication of CLRV in ***N. rustica*** cultures. In the present work, SLRV may be completely eradicated in seed cultures of the same host after 28-36 days treatment at 33°C.

Strawberry latent ringspot virus did not reappear in ***N.rustica*** seed cultures after being transplanted in Levington compost and grown in normal glasshouse temperature (20-27°C) for about two months. With parsnip cultures, the virus was detected (by ELISA) in about 40% of plants after three to four months. In view of this, longer treatment (e.g 50 days at 33°C or 35°C) may be required for complete eradication of SLRV in parsnips.

There are apparently no previous reports of the eradication of SLRV by thermotherapy in tissue cultures. ***In vitro*** culture combined with thermotherapy seemed to be a satisfactory method for the production of virus-free plants, in these studies. Such plants could provide a nucleus of virus-free seed mother plants, provided precautions were taken to avoid other sources of infection. Production of seed under warmer climatic conditions may further minimise the incidence of infection, although the quality and quantity of seeds produced under high temperature should be carefully monitored.

In contrast to thermotherapy, chemotherapy of SLRV in seed cultures of ***N.rustica*** and ***P.sativa*** was less successful. The lower ribavirin concentrations tested (0.1 - 10 mg/ l) apparently eliminated SLRV from about 24% of parsnip cultures and about 10-20% of ***N.rustica*** cultures, but higher levels (100 mg/ l) had no effect on the number of virus-free cultures produced. There was, however, no evidence of the phytotoxicity sometimes reported with this chemical

(Kartha, 1986). It is possible that at 100 mg / l ribavirin decreased cell resistance, as well as inhibiting virus multiplication, whereas lower levels inhibited virus synthesis only. A more prolonged exposure to ribavirin, may have increased the proportion of virus-free cultures produced. Long and Cassells (1986) working with cucumber mosaic virus in tobacco explants reported that a six week exposure of cultures to ribavirin (250 µM) produced only 28% virus-free plants compared to 100% virus-free for continuous exposure.

Only one out of 17 parsnip plants in the field tested for virus was infected with parsnip yellow fleck virus (PYFV) although it was the commonest virus to infect parsnip in Britain (Murant and Goold,1968). The virus was distinguished from SLRV by its reaction in ***C. quinoa*** and was confirmed by serology.

The isolate (PYFV-E) obtained from parsnip produced systemic mottle and ringspotting in ***N.clevelandii*** typical of the parsnip strains of PYFV (Murant and Goold, 1968); the ***Anthriscus*** strain of PYFV does not normally go systemic in ***N. clevelandii*** . Furthermore, the host range of PYFV-E was similar to those of PYFV isolates described by Hemida and Murant (1989) although differences in symptom expression were found in some hosts. For example PYFV-E systemically infected ***C.amaranticolor*** with leaf distortion while P-121 (an isolate from parsnip, Murant and Goold, 1968) infected inoculated leaves only. No other PYFV

isolate apparently induced systemic reaction in *C. amaranticolor* except a celery isolate (Singh and Frost, 1987 ; Lennon,1984). Moreover, PYFV-E was systemic in *N. tabacum* 'White Burley' where all other isolates described so far induced local reaction only. A comparison of host range of PYFV-E and P-121 strain (Hemida and Murant, 1989) is given in the Appendix (Table 3).

The physical properties of the PYFV-E were similar to those reported by Murant and Goold (1968) for their isolates of PYFV.

The PYFV-E was partially purified with butan-1-ol clarification, but recovery was poor after passage through the CPG column. However, after two cycles of differential centrifugation the partially- purified preparation showed a moderate number of particles in the electron microscope and high infectivity when assayed on *C. quinoa*.

The partially purified preparation had A_{280}/A_{280} ratio of 1.6-1.8 and isometric particles with smooth round surface when seen under the electron microscope. When stained in 2% *dodeca*-tungstophosphoric acid, some particles were seen penetrated by stain. Stain penetrated particles are thought to be 'empty' protein shells and were observed by Hemida and Murant (1989) in preparations clarified by butan-1-ol but not in preparations purified using butanol/chloroform (Murant and Goold, 1968). Characteristics of the uv absorption spectra of P-121 (Hemida and Murant,1989), PYFV-E and the angelica isolate

of PYFV (AV1) are given in the Appendix (Table 4).

The preparations of P-121 clarified by di-ethyl ether or butan-1-ol subjected to sucrose density gradients, resulted in two components (Hemida and Murant,1989). The upper zone (T components) contained only particles that were penetrated by negative stain ('empty' shells) whereas the bottom zone (B components) contained only 'full' particles not penetrated by stain. In the present work, after two cycles of differential centrifugation, unfractionated particles had A_{280}/A_{280} ratio between 1.60 -1.80 with very few 'empty shells' and were used in antiserum production. The antiserum prepared using these preparations, however, had a DEP of 1/8 when tested against healthy crude sap although this was reduced by cross absorbance against a host protein preparation.

PYFV-E yielded particle proteins that contained three electrophoretic species. A comparison of estimated relative molecular masses of particle proteins of PYFV-E ,P-121 and A-421 isolates (Hemida and Murant,1989) are given in the Appendix (Table 5).

Several attempts to extract dsRNA of PYFV by phenol/SDS method (Dodds ***et al.***,1983) were not successful. Systemically-infected leaves of spinach or ***N.clevelandii*** with well-established symptoms were used for the extractions and may have contained relatively low levels of virus and dsRNA. Evidence that harvesting time can be critical for optimum yields come from SLRV in ***C.quinoa*** where a lower level of dsRNA

was extracted from leaves 12 days after inoculation, compared to six or nine days. Time of harvest was found to be critical in the detection of dsRNA from lettuce roots infected with lettuce big vein (Mirakov and Dodds , 1985). In future experiments leaf tissues should be sampled within one or two weeks of inoculation before the full expression of symptoms.

In reciprocal gel-diffusion tests, both PYFV-E and AV1 reacted with homologous and heterologous antisera without forming obvious spurs suggesting that the two islates were closely related serologically. Moreover, they both belonged to P-121 serotype (Murant and Goold,1968 ; Hemida and Murant, 1989) according to the reaction on ***N. clevelandii.*** However, isolates which belonged to P-121 serotypes were reported to be serologically related but not identical (Hemida and Murant,1989). Two isolates from carrot, however, which belonged to A-421 serotype were reported to be serologically identical.

In immunoelectrophoresis experiments, PYFV-E (and AV1) migrated towards the anode and produced a single precipitin line with the homologous antiserum. Similar results were reported by Murant (1974) for his isolates of PYFV. Experiments in which the parsnip and angelica isolates were compared, showed that the two isolates differed slightly in electrophoretic mobility.

PYFV-E was returned to healthy parsnip using a partially-purified preparation although, only two out of seven

manually inoculated plants became infected. Infected plants developed a slight chlorotic mottle five weeks after inoculation. The reaction was less severe than that described by Murant (1974) probably because of cultivar variability.

N.clevelandii and ***C.quinoa*** plants doubly infected with PYFV-E and SLRV-D or RRV developed more severe symptoms than plants infected with either virus alone. The increase in symptom severity was associated with increased concentration of PYFV-E of SLRV-D as determined by infectivity assay and ELISA. Similar findings were reported by Barker (1986) in ***N.clevelandii*** plants infected with potato leafroll virus (PLRV) and potato virus Y (PVY). This author reported that PLRV concentration was increased up to eightfold by the double infection with PVY, although the concentration of PVY was not affected. Similarly, the synthesis of PVX was enhanced by double infection with PVY and TMV in tobacco (Rochow and Ross, 1955; Goodman and Ross,1974). More recently, Barker (1989) reported that some viruses belonging to the potyvirus and tobravirus groups, narcissus mosaic potexvirus and carrot mottle virus enhanced the synthesis of PLRV in mixed infections. This did not occur when PLRV was present with nepoviruses, cucumber mosaic virus, alfalfa mosaic virus, brome mosaic virus or PYFV. In the present study, although PYFV synthesis was apparently enhanced by SLRV the concentration of SLRV was not affected. However, the concentration of SLRV was only estimated once.

Parsnip yellow fleck virus was found to be seed transmitted in ***N. clevelandii*** both as a mixed infection with SLRV and in single infections. In mixed infections, PYFV was occasionally detected with SLRV in progeny seedlings, but both viruses could also be transmitted independently to seedlings indicating that SLRV did not act as a 'helper' virus for PYFV during seed transmission of PYFV. Furthermore low levels (10-30%) of seed transmission of PYFV were detected in singly infected mother plants. This report, however, is apparently the first record of seed transmission of PYFV although experiments are needed to see if the virus is also transmitted through parsnip seeds.

Parsnip yellow fleck virus transmission is dependent on the 'helper' Anthriscus yellows virus (AYV) (Murant and Goold,1968) but if the host is immune to AYV, spread depends whether the aphid vector has previously acquired AYV. For example, PYFV cannot be spread from parsnip to parsnip or from carrot to carrot (Murant and Goold, 1968) in the field by the vector, as these species are immune to AYV. In addition, the host range of AYV is limited,and the only hosts found by Murant and Goold (1968) were ***Anthriscus sylvestris*** (cow parsley), ***A.cerefolium*** (chervil) and coriander. In Britain, hog weed (***Heracleum spondylium***) was reported to be a major source of PYFV (Tomlinson and Carter, 1970 ; Bem and Murant, 1979), which was also susceptible to AYV. In view of the rather limited (vector-dependant) spread of PYFV in the field, seed transmission would provide an

important potential reservoir of infection. Transmission through wild plants could provide an important overwintering source of inocula particularly if such plants were susceptible to AYV. *N. clevelandii* seedlings infected with PYFV were symptomless but the use of sensitive assay procedures like ELISA, should facilitate surveys needed to demonstrate natural seed transmission of PYFV in umbelliferous crops.

There are no report on the effects of PYFV infection on parsnip yield. In future work, therefore, it would be interesting to know whether infection affected the quality or quantity of parsnip seeds and roots . Although there was no evidence that the PYFV or SLRV affected parsnip root yield, recent work has suggested other serious consequences of infection. Lord *et al.*, (1988) found that PYFV infection in celery increased the production of toxic furanocoumarins which are thought to be carcinogenic.

Both PYFV and SLRV infection can be controlled to some extent by eliminating their vectors. Soil-borne infection of SLRV on nurseries can be reduced by fumigating with nematicides, although Ikin and Frost (1976) reported only a low incidence of SLRV infection among rose seedlings planted for a year in soil cotaining viruliferous *Xiphinema diversicaudatum* . Sweet *et al.*, (1978) also reported that only one out of five rose bushes became infected after two years growth in viruliferous soil. Furthermore, in a survey of 2000 soil samples collected throughout Britain (1969-1973) *X.*

diversicaudatum occurred in 325 but only 18 of the populations were shown to be viruliferous (Taylor and Brown, 1976). Nevertheless, in the present work nematodes efficiently transmited SLRV to both parsnips and *C.quinoa*, and it is probable that the infection in parsnip cv. White Gem and Tender and True, arose in this way.

The aphid vectors of PYFV may be reduced by spraying with systemic insecticide although the effectiveness of such chemicals against virus transmission is limited, since aphids may transmit virus before they are killed. Other approaches such as mineral oil sprays (Pirone and Harris, 1977) could be explored but the spread of PYFV in the field, anyway, may be limited by the availability of AYV helper. It would be important to know , however, if viruses other than AYV can act as helpers. Many different viruses, for example, can act as helper for the dependent potato aucuba mosaic virus (Kassanis and Govier, 1971).

Virus control may also be achieved by destroying alternative sources of vector and/or virus. Thus, removing cow parsley and hog weed would be an effective means of controling PYFV infection, where these were the main source of infection. Similarly, wild hosts of SLRV (Schmelzer, 1969) should be removed regularly from nursery sites as these may act as reservoirs of virus and vectors.

The discovery that PYFV can be seed-borne in some hosts, has important implications for the ecology and

epidemiology of the virus. If seed transmission can be confirmed in natural hosts it would provide PYFV with additional means for survival and spread in the field. Studies on the longevity of virus in seed, however, need to be done, and it would be interesting to know if the helper AYV,is seed-borne, especially where it occurred with PYFV.

Seed-borne infection was an important source of SLRV in parsnip, and may be for PYFV. The advantages of using virus-free seeds are obvious, particularly if there is also efficient vector transmission. With SLRV in parsnip cv. White Gem,where infection of seed stocks has been widespread (Cooper, 1981; Hicks ***et al.***, 1986; this study), it may be worth re-establishing fresh supply of seeds from virus-free mother plants. As shown in the present work, tissue culture techniques, combined with thermotherapy or chemotherapy, proved useful for eliminating virus from seeds or seedlings. However, strict crop hygiene must be maintained to avoid subsequent reinfection of mother plants, and the virus status regularly monitored (eg. by ELISA) to ensure the crop remains virus-free.

CHAPTER 11 References

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APPENDIX

Table 1 A comparison of the physical properties of SLRV isolates from parsnip

Isolate	DEP	TIP °C	LIV (days)
Cooper (1981)	10^{-3}-10^{-4}	55	NR
Van der Put(1981)	10^{-5}-10^{-6}	60	8
Hicks *et al.*, (1986)			
mild isolate	10^{-3}-10^{-4}	55-60	32-64
severe isolate	10^{-2}-10^{-3}	50-55	8-16
SLRV-R (mild)	10^{-3}-10^{-4}	55-57	8-16
SLRV-D (interm.)	10^{-2}-10^{-3}	57-59	8-12
SLRV-I (severe)	10^{-2}-10^{-3}	55-60	4-8

Table 2 Main differences in host range and reaction between SLRV isolates from parsnip (Smith, 1986) and strawberry (Lister, 1964)

	Lister (1964)	Smith (1986)	SLRV-D	SLRV-R
Chenopodiaceae				
C. quinoa	LS	S	(L)S	(L)S
C.foetidum	NR	(S)	LS	LS
Bete vulgaris	NR	-	LS	LS
Solanaceae				
Datura stramonium	(S)	-	-	-
N. clevelandii	(S)	(S)	(S)/S	(S)/S
N.tabacum 'White Burley'	(S)	-	(S)	(S)
N. sylvestris	(S)	-	(S)	(S)
Umbelliferae				
Daucus carota	(S)	-	(LS)	(LS)
Leguminoseae				
P.vulgaris	LS	-	-	-

L, local infection
S,systemic infection
(), letters in parenthesis indicate symptomless infection
-, no infection
NR,not reported

Table 3 A comparison of host range of P-121 (Murant and Goold, 1968) and PYFV-E

Plant species	P-121	PYFV-E
Chenopodiaceae		
C.quinoa	LS	LS
C.amaranticolor	L	LS
C.murale	L	NT
C.foetidum	NT	LS
Spinacea oleracea	LS	LS
Cucurbitaceae		
Cucumis sativus	I	L
Leguminaceae		
Phaseolus vulgaris	0	0
P. multiflorous	NT	L
Vicia faba	0	0
Solanaceae		
N.clevelandii	LS	LS
N.tabacum 'White Burley'	L	LS
N.tabacum 'Xanthi'	NT	LS
N.glutinosa	L	(S)
Datura stramonium	I	0
Umbelliferae		
Coriandrum sativum	LS	LS
Daucus carota	LS	0
Anthriscus cerefolium	LS	LS
Apium dulce 'Lisbonais'	LS	NT
Apium graviolens	NT	LS
Pastinaca sativa	LS	LS

L, local reaction
S, Systemic reaction
0, No infection
NT, Not tested
I, Rare local reaction
(), Symbols between parenthesis indicate symptomless infection

Table 4 A comparison of UV absorption spectra of P-121 (Hemida and Murant,1989), PYFV-E and AV1.

	P-121		PYFV-E	AV1
	B	T	(unfractionated)	
Maximum (nm)	259	277	258	259
Minimum (nm)	239	249	239	239
A_{280}/A_{280}	1.59	0.78	1.6-1.8	1.6-1.85

B, bottom component T, top component

Table 5 A comparison of estimated Mr between P-121, A-421 and PYFV-E

Isolate	No of determinations	Mr ($x10^{-3}$) Mean	S.D
A-421	24	30.1	0.7
		25.8	0.7
		23.8	0.5
P-121	17	30.9	0.8
		25.5	0.8
		22.7	1.0
PYFV-E	4	36.0	1.4
		29.0	0.71
		22.0	1.4